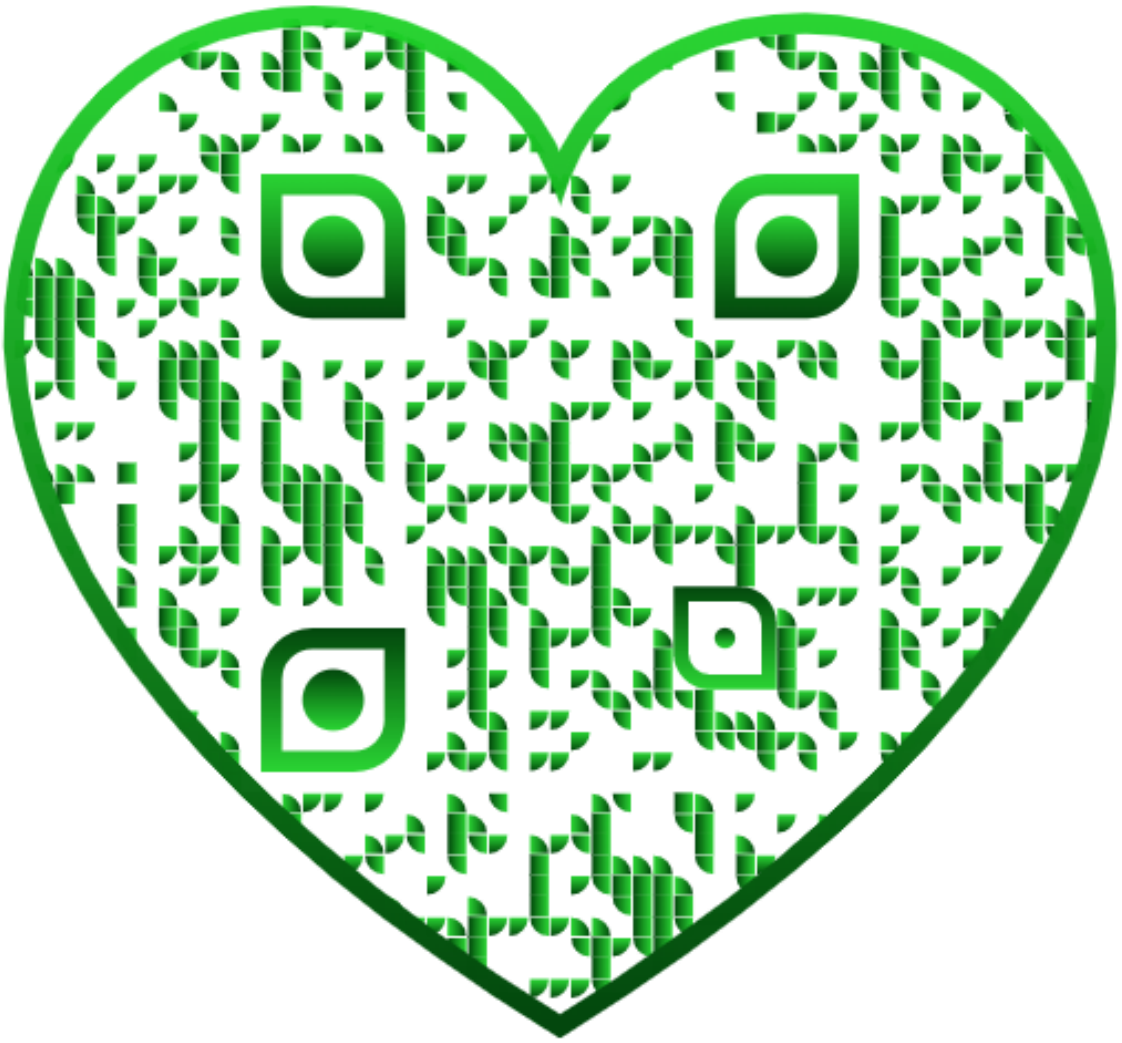
Edible and Medicinal Mushrooms



Edible and Medicinal Mushrooms

Technology and Applications

Edited by

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WILEY Blackwell

This edition first published 2017

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9600 Garsington Road, Oxford, OX4 2DQ, UK
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Library of Congress Cataloging-in-Publication Data applied for

9781119149415

Cover design by Wiley
Cover image: Courtesy of Antonio Martínez Carrasco

Set in 10/12pt WarnockPro by SPi Global, Chennai, India

10 9 8 7 6 5 4 3 2 1

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Acknowledgments

We would like to thank all the people who contributed to the development of the chapters that make up this book and, in particular, to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) in Brazil and the Patronato de Desarrollo Provincial of the Diputación de Cuenca and the Consejería de Agricultura, Medio Ambiente y Desarrollo Rural of Castilla-La Mancha in Spain, for their financial support for our research and allow for the advancement in science.

Preface

The term *Mushrooming*, or mushroom cultivation, refers to the intentional and directed production of mushrooms as a substitute for wild collection in fields and forests with a harvest under defined conditions of growing, resulting in strict quality control and food safety without risk of consumption of poisonous or toxic species, and with guaranteed benefits from fungi.

Although knowledge about the cultivation of edible and medicinal mushrooms is practically the same throughout the world, there are significant differences between countries and even within the same country. These are primarily associated with different socioeconomic conditions. In this way, just as there are large-scale growers, other smaller-scale plants act as a complement to the family economy, while very basic and rustic facilities coexist with others that operate on a high technological level.

This book involves a multidisciplinary approach that includes aspects of agriculture and agronomy, microbiology, biology, biotechnology, chemistry, environmental management, food technology, and health, among others. With a global and collaborative purpose, the book consists of 22 chapters written by 28 authors, from 15 different countries, who are recognized experts in the different areas that compose this activity. We thank them all for their participation.

The different areas of the science of cultivation are approached, so the book can serve as a tool for researchers, professors, technical specialists, and growers, and as an introduction for both students and anyone interested in the world of *mushrooming* knowledge as a business opportunity or out of simple curiosity.

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1

Mushrooms and Human Civilization

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Mention of mushrooms has been reported in ancient literature since the inception of human civilization. Mushrooms find mention because of their wide range of properties from being poisonous to being beneficial and edible. Their poisonous nature was their most intriguing quality in early history. Throughout the centuries, poisonous fungi/mushrooms have remained a useful means of disposing of adversaries. Pliny the Elder (23–79 AD) gives details of how the Emperor Claudius was poisoned by his fourth wife Julia Agrippina. Emperor Jonan followed in 364 AD, and Pope Clement VII in 1394. In addition, the antipope Urban VI, the French King Charles VI, and the German/Spanish king Joseph Ferdinand were all poisoned with mushrooms (van Griensven, 1988).

Knowledge about fungi developed slowly. In the fourth century BC, Theophrastus gave a scientific description of fungi and considered these fungi as part of vegetable kingdom, even though they have no buds, leaves, or roots.

With the decline of classical civilization, interest in science also declined. The scholastics of the Middle Ages made no contribution to science. Scientific study made little progress in the Western World up until the late Middle Ages. Names were given, morphological descriptions were made, and mushrooms find mention as "surplus moisture from the ground and trees, from rotting wood and other things." This particularly applies to edible varieties, through the influence of thunder, lightning, and rain (van Griensven, 1988).

In China, however, as early as 1245 AD, Chen Yen-Yu had published a fungus flora, describing in detail the development, morphology, seasonal influence, growing method, harvesting, and preparation (as food) for 15 varieties of mushrooms (Wang, 1987). In 1588 Giambattista Porta published his *Phytognomoniica*. He was the first person to describe the spores of fungi. Like his contemporaries, he held the view that parasitic plants, among which he counted tree mushrooms, were unnatural and could be used against lumps and tumors on human limbs (van Griensven, 1988).

According to Theophrastus, practically everything was missing from the mushroom, and eating mushrooms was therefore harmful to human body. Clausius (1525–1609) was the first to describe the Bird's Nest (Nidularia).

The "hidden power" of earth is responsible for the occurrence of mushrooms. That is why mushrooms were known as "excrementa terrae" in the seventeenth century. It was, of course, reprehensible to eat these excretions of the earth.

In the early seventeenth century, the Italian Count Margigi describes how a white, mold-like web appears when mushrooms and truffles are carefully dug up. He calls this web, which smells

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Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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of mushrooms and has tiny buds, "situs" (Lutjehmas, 1936). By this time all edible mushrooms including truffles were found in Europe, collected from the wild.

The Chinese and the Japanese were probably the first to cultivate mushrooms professionally, and a brief description of history published in English (Wang, 1987) refers to Shiitake mushroom cultivation by Wang Zeng in 1313 AD. The culture of the paddy straw mushroom *Volvariella volvacea* is also centuries old.

Linnaeus (1707–1778) gave the field mushroom (white button) the name *Agaricus campestris*. Finally, in his *Systema Mycologicum* (Kleiju, 1961; Poppe, 1962), Elias Fries (1707–1778) gave a methodical description of all varieties of mushrooms known at that time (van Griensven, 1988).

1.1 Domestication of Mushrooms

The mushroom is the most important horticultural cash crop grown indoors, compared to other traditional crops grown outdoors, and is the only non-green crop grown for commerce with attractive profits. Mushroom is the fruit body of a fungus, which is neither a plant nor an animal, but has a separate kingdom of its own. Fungi as a broad group either live parasitically on plants and animals or live saprophytically on dead organic matter. Fungi cause numerous diseases of plants and animals and have been reported to cause considerable crop losses with tremendous suffering to mankind from time immemorial. The role of fungi as being beneficial to humans is of recent origin, with the generation of information on existence of microorganisms and their importance to man on Earth. Today, the science of study of mycological applications for human welfare has touched greater heights with the application of molecular biological techniques to improve useful fungal cultures of yeasts and mushrooms.

The fact that certain fungi are edible has been known for many centuries, and in various European countries up to 80 distinct varieties of wild fungi are offered for sale on the market (Pinkerton, 1954). Though many edible fungi have been domesticated and are in production, the most commonly cultivated are shiitake (*Lentinula edodes*), oyster mushroom (*Pleurotus* spp.), white button mushroom (*Agaricus bisporus*), black fungus or wood-ear mushrooms (*Auricularia auricula and Auricularia polytricha*) and paddy straw mushroom *Volvariella* spp. The cultivation of shiitake by Japanese on logs dates back at least 2000 years (Ainsworth, 1976), but button mushroom cultivation is comparatively recent. Today, the button mushroom is the most widely grown in many countries, although it is the fourth mushroom most produced in quantity (see chapter 2), with most of the development of cultivation technology confined to improving this mushroom for reasons of its larger acceptability by the consumer.

The first record of (button) mushroom cultivation dates back to Abercrombie (1779), who wrote that this plant is of so very singular growth and temperature, that unless a proper idea of its nature and habit is attained, and the peculiar methods and precautions pursued in the process of its propagation and culture, little success will ensue; the whole management of it differs remarkably from that of every other species of the vegetable kingdom; and it is the most liable of any to fail without very strict observance and care in the different stages of its cultivation.

Tournefort (1707) gave a comprehensive description of the commercial production of button mushrooms. These observations recorded in earlier times bear comparison with the methods used today. At that time mushrooms were cultivated on open ground, but around 1810, Chambry (a French gardener) began to cultivate mushrooms in underground quarries in Paris, all year round. Later Callow (1831) showed that mushroom production was possible all year round in England in rooms specially heated for the purpose. Callow gave details of the design of cropping houses (crediting it to Oldacre, a garden superintendent in UK) and later successfully grew mushrooms all year round in such a structure producing a yield of 7.3 kg m^{-2} in

24 weeks of cropping, as compared to mushroom yields of 10 kg m^{-2} obtained in 1950 in the UK. It is now accepted that protected cropping of mushrooms was pioneered in caves in France, though the earliest mushroom houses were developed in England.

Large-scale mushroom production is now centered in Europe, North American (USA, Canada), Australia, South East Asia (China, Korea, Indonesia, Taiwan), and South Asia (India). The notable contributions to mushroom science in recent times were made at the beginning of the twentieth century when pure cultures of button mushrooms were grown by Duggar (1905). Other notable contributions were the preparation of mushroom compost from agro-byproducts using the short method by Sinden and Hauser (1950, 1953).

Contributions by Fritsche (1985) in breeding two new strains of white button mushroom *A. bisporus* U-1 and U-3 revolutionized commercial mushroom growing across the world. With the refinement of cultivation technology of button mushrooms on a continuing scale, it was possible to harvest more and more quantities of mushrooms per unit area/unit weight of compost. Demonstration of steam pasteurization of mushroom compost in bulk (Derks, 1973) further helped commercial mushroom growing to increase the productivity per unit area/unit weight of compost.

Finally, increased understanding of crop management techniques resulted in substantial increases in mushroom yields per unit weight of compost in a reduced cropping period, thereby giving greater profitability to the mushroom grower. Today, mushroom growers worldwide have a wide range of button mushroom cultivars available for cultivation. Computer control of cropping room environments for climate creation/simulation has made it possible to harvest mushroom yields of 30–45 kg from 100 kg compost within a cropping period of 3–4 weeks in 2–4 flushes.

With the introduction of the use of phase-I aerated bunkers for environmental protection, the composting process has become precision controlled with reduced emission of foul harmful gases without affecting mushroom yield. Use of indoor aerated bunkers has become very popular all over the world for reasons of economy in addition to being environmentally friendly. Phase-I bunkers are less space demanding and less labor oriented than traditional outdoor phase-I ricks, with the advantage of lower emission of foul gases during solid state fermentation controlled by restricted/controlled oxygen availability in the bunker.

A current science of mushrooms is presented in detail in this book, along with specific approaches in the main species of cultivated mushrooms and their technologies in different countries and continents. All steps and applications of "mushrooming" are detailed in the following 21 chapters.

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2

Current Overview of Mushroom Production in the World

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Edible, medicinal, and wild mushrooms are the three major components of the global mushroom industry. Combined, the mushroom industry was valued at approximately $63 billion in 2013. Cultivated, edible mushrooms are the leading component (54%) accounting for approximately $34 billion, while medicinal mushrooms make up 38% or $24 billion and wild mushroom account for $5 billion or 8% of the total (Figure 2.1).

World production of cultivated, edible mushrooms has increased more than 30-fold since 1978 (from about 1 billion kg in 1978 to 34 billion kg in 2013). This is an extraordinary accomplishment, considering the world's population has increased only about 1.7-fold during the same period (from about 4.2 billion in 1978 to about 7.1 billion in 2013). Thus, *per capita* consumption of mushrooms has increased at a relatively rapid rate, especially since 1997, and now exceeds 4.7 kg annually (vs 1 kg in 1997; Figure 2.2).

In 2013, nearly all consumption of mushrooms in China, EU, and India was supplied from domestic sources; and nearly all consumption of mushrooms in the United States, Canada, Japan, and Australia was supplied mostly by domestic sources but also by substantial amounts of imports (USITC 2010).

China is the main producer of cultivated, edible mushrooms (Figure 2.3). Over 30 billion kg of mushrooms were produced in China in 2013 (CEFA, 2014) and this accounted for about 87% of total production. The rest of Asia produced about 1.3 billion kg, while the EU, the Americas, and other countries produced about 3.1 billion kg.

Five main genera constitute around 85% of the world's mushroom supply (Figure 2.4). *Lentinula* is the major genus, contributing about 22% of the world's cultivated mushrooms. *Pleurotus*, a close second, with five or six cultivated species, constitutes about 19% of the world's output while *Auricularia* contributes around 17%. The other two genera, *Agaricus* and *Flammulina*, are responsible for 15 and 11% of the volume, respectively.

Edible mushroom production in China by genus in 2013 is shown in Figure 2.5. *Lentinula* is the most widely grown mushroom accounting for over 7 billion kg. This represents a 106.8% increase in volume from 2010 (Figure 2.5). The second most widely grown mushroom in China is now *Auricularia*. Production of this genus (with two main species) has increased nearly 92% since 2010. *Pleurotus* is the third most widely grown genus in China 2013 accounting for nearly 6 billion kg (a 10.8% increase since 2010).

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Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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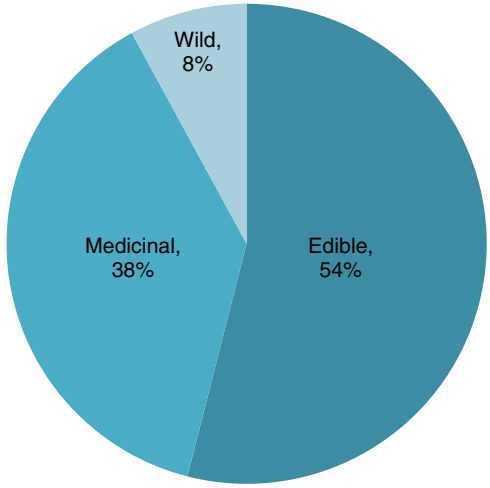


Figure 2.1 Components (edible, medicinal, and wild) of the world mushroom industry based on percentage of total value ($63 billion) (2013).

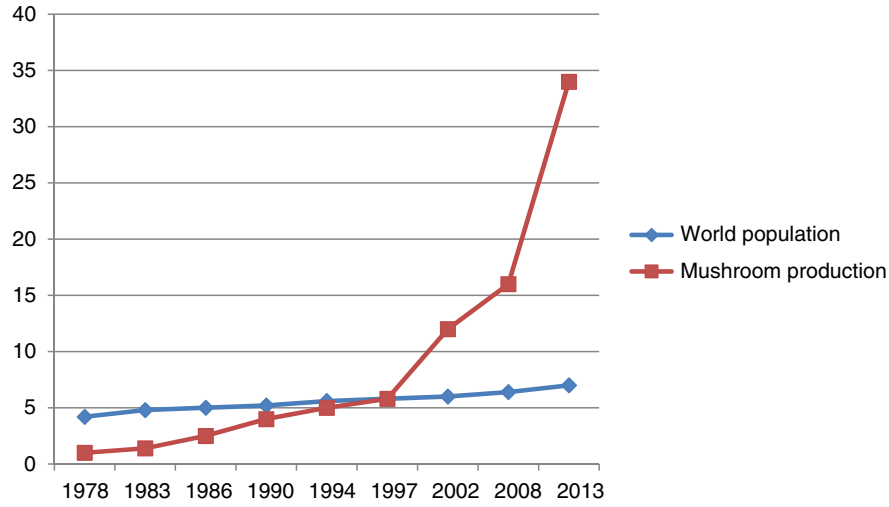


Figure 2.2 World population (billions) versus world cultivated, edible mushroom production (billion kg).

2.1 *Lentinula edodes*

Until the mid-1980s, Japan was the world's major producer of *L. edodes* (shiitake in Japanese or xianggu in Chinese) (Figure 2.6) that were grown on natural logs of the shii tree (*Castanopsis cuspidata*). However, with the development of sawdust-based techniques (Figure 2.7) that reduced crop cycle time and increased production efficiency, China soon became the major producer of xianggu by 1990 (Figure 2.6). From 1995 to 2000, Chinese farmers increased xianggu production from about 500 million kg to over 2 billion kg – a huge increase by most standards of measuring change. China now accounts for more than 95% of total output of this species. Several entire communities have been lifted from poverty because of the economic opportunities afforded to them by producing xianggu (Chang 1999, 2005).

Production of dried shiitake in Japan has been steadily declining since the early 1980s (Yamanaka 2011). During the 10-year period from 2000 to 2009, dried shiitake production

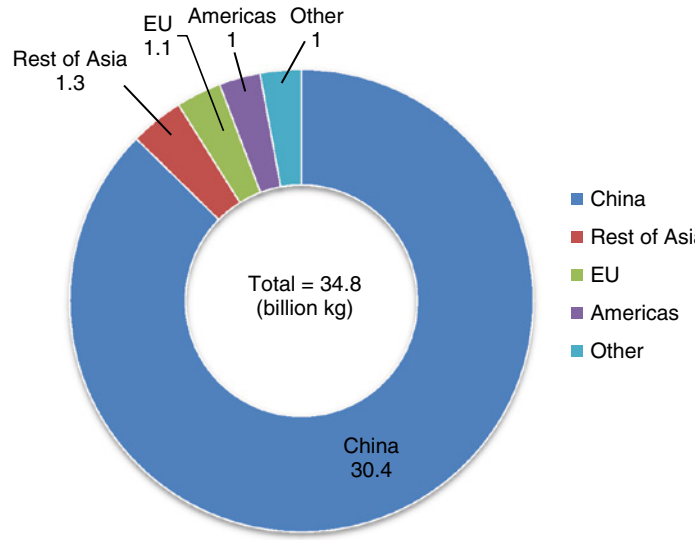


Figure 2.3 Cultivated mushroom production in China and selected regions of the world, 2013 (billion kg).

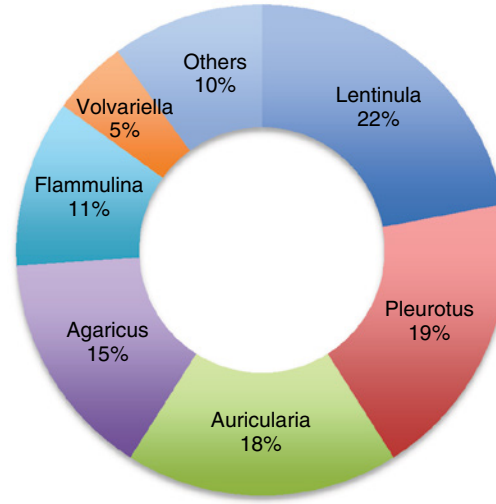


Figure 2.4 World edible mushroom production (% of total) by genus (2013).

declined by 37% while fresh shiitake production increased by 12%. Production increases for fresh shiitake were due to fulfilling the demand left by decreased imports of fresh shiitake from China. Total production of *L. edodes* (based on fresh mushrooms plus dried mushrooms converted to fresh equivalent) was slightly over 101 million kg in 2009, which ranked third with 22% of total production of edible mushrooms in Japan.

In the United States, most shiitake production is on nutrient-supplemented, sawdust-based substrates (Royse 1997, 2009, 2013, 2014). Many growers use a 16–20-day spawn run then remove the bag for browning of the exterior surface ("skin") of the "log" while other growers conduct spawn run and browning inside the bag. In general, higher rates of supplements may be used when logs are browned outside the bag resulting in higher yield potential compared to logs browned inside the bag. Over the last 10 years, shiitake production in the United States has increased by 24% (from 3.64 million kg in 2006 to 4.78 million kg in 2015) (USDA 2015). In recent years, sawdust-based logs made in China have been imported into the United States and

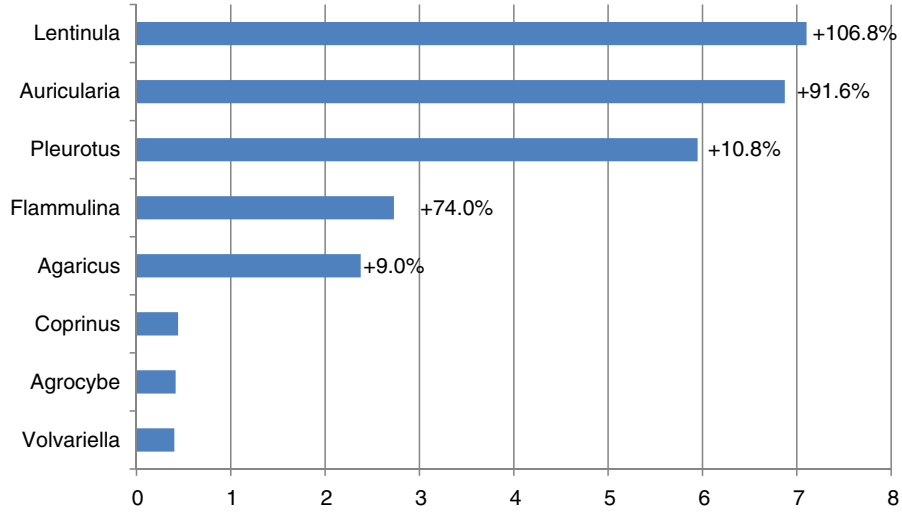


Figure 2.5 Mushroom production in China by genus (2013, CEFA 2014). Percentages following horizontal bars for each genus represent change from 2010 production levels (in billion kg).

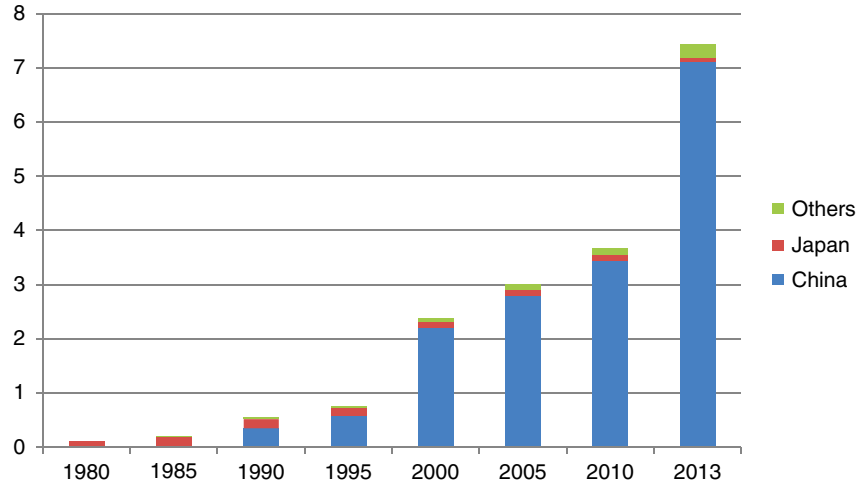


Figure 2.6 Growth in world shiitake production (1980–2013; billion kg).

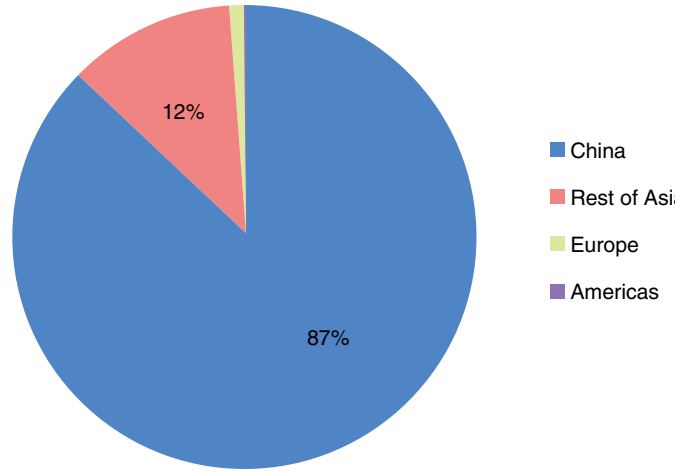
these logs have begun to gain traction with growers because of the relatively low cost and excellent mushroom quality (Figure 2.7).

2.2 *Pleurotus* spp.

Asian countries (especially China, Japan, South Korea, Taiwan, Thailand, Vietnam, and India) are the main producers and consumers of oyster mushrooms with approximately 99% of the total volume (Figure 2.8). China is the main producer with 87% of total world production of these species. Most of China's oyster mushrooms are from two species: *P. ostreatus* and

Figure 2.7 Sawdust-based "logs" of *Lentinula edodes*: (left) arranged in rows under shade cloth-covered shelters and (middle and right) with maturing mushrooms (photos: D. J. Royse and Q. Tan).

Figure 2.8 Percentage of total world *Pleurotus* spp. production in selected countries and regions.



P. cornucopiae. In the last 5 years or so, however, substantial increases in production of *P. eryngii* and *P. nebrodensis* have occurred. In China, administrative and professional agencies have developed plans to help guide growers in their initial selection of regions where production and utilization of resources may be optimized for mushroom production. The middle regions of China, especially the provinces of Henan, Hebei, and Shandong, are the major production areas for *Pleurotus* spp.

In Japan, production of *Pleurotus* spp. increased nearly 200% from 1997 (13.3 million kg) to 2010 (39.6 million kg). *Pleurotus eryngii* experienced the largest gains in production, in terms of percentage (+453%), increasing from 6.7 million kg in 2000 to over 37 million kg in 2009 (Yamanaka 2011). Most *P. eryngii* is cultivated on sawdust of Japanese cedar or ground corncobs supplemented with bran and contained in polypropylene bottles.

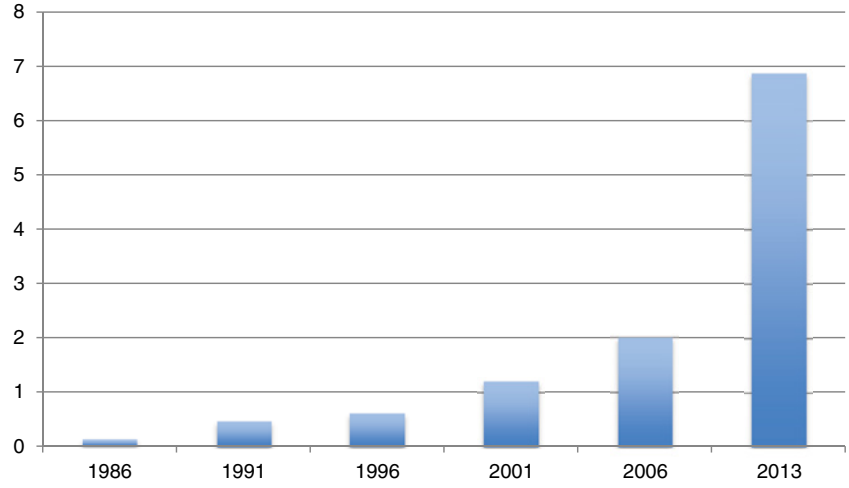


Figure 2.9 Growth in world *Auricularia* spp. production (billion kg) (1986–2013).

2.3 *Auricularia* spp.

Black fungus or wood-ear mushrooms (mostly *A. auricula* and *A. polytricha*), now widely cultivated in China, Taiwan, Thailand, Philippines, Indonesia, and Malyaysia, are considered the earliest cultivated mushrooms (Tang et al. 2010). Wood-ear production accounts for about 18% of the world's total output of mushrooms (Figure 2.4). Annual production of *Auricularia* spp. in China alone reached nearly 6.9 million kg in 2013, making them the second most widely cultivated mushrooms in that country (Figures 2.5, 2.9; CEFA 2014). Production figures for 2013 for this genus represent a 91.6% increase over 2010 figures.

Successful domestication of wild-type strains over an extended period of time by farmers in the Changbaishan and Shennongjia regions of China has led to rapid growth in production of these species. Some of the domesticated strains now have been introduced to new cultivation regions located in Northern and Southeastern regions of China.

2.4 *Agaricus bisporus*

China is the number one producer of *Agaricus bisporus* accounting for 54% (2.37 billion kg) of the world's total production of this species in 2013 (Figure 2.10). The USA produced about 9% (409 million kg) of the world's total followed by Poland (285 million kg), the Netherlands (270 million kg), and India (250 million kg).

In the last few years, production of *A. bisporus* in China has gradually moved northward as climatic conditions in the northern provinces are more conducive for mushroom production and raw materials are more readily available compared to southern provinces. This trend is expected to continue for the next few years (Li 2012). In the United States, production has increased about 11.7% over the last 10 years (from 378.9 million kg in 2006 to 423.2 million kg in 2015) (USDA 2006, 2015). Growth in production of the white variety has increased 10.1% while the brown variety (portabella and crimini) has increased 24.3% over this 10-year period.

Production of *A. bisporus* in Europe continues to move eastward (Lelley 2014; Royse 2014). Poland has become the world's third largest producer, outstripping the Netherlands by

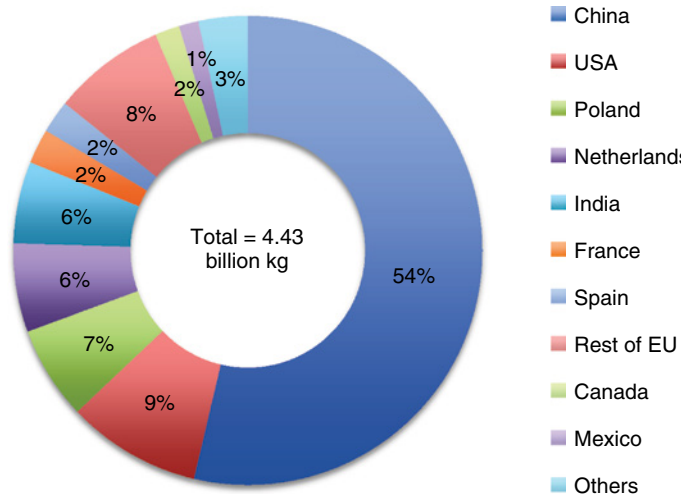


Figure 2.10 Production of *Agaricus bisporus* in selected countries and regions (2013).

approximately 9 million kg in 2013. This gap widened even further in 2014 with Poland producing 315 million kg while the Netherlands held steady at 270 million kg. Many Dutch-style farms have been constructed recently in Poland – especially in the eastern part of the country (Bieniecka and Dreve, 2012; Rozendaal 2012). Production output in Poland has recently become uncertain due to the conflict in the Ukraine and to the fact that approximately 90% of the Russian market was supplied by Poland.

In the Netherlands, the fourth largest producer of *A. bisporus*, over 90% of production is in the southeastern part of the country, that is, in the providences of Limburg, Brabant, and Gelderland (Baars 2012). Approximately 90% of the crop is exported as either frozen or canned (60%) while nearly 30% is exported as fresh mushrooms. The UK consumed about 41% of the fresh supply while France, Germany, Belgium, Norway, and Sweden bought most of the remainder of the fresh mushrooms (Baars 2012, Royse 2014).

2.5 *Flammulina velutipes*

Until the mid-1990s, Japan was the dominant producer of this species. Then, beginning in about 1997, China became the world's largest producer of *F. velutipes*. Production has increased from about 0.12 billion kg in 1995 to about 2.7 billion kg in 2013 (Figure 2.11).

In the last 10 years or so, many new enoki farms, based on bottle technology, have been constructed in China. In a description of one recent new enoki farm in China, Dreve (2014) describes the first stage of a large climate-controlled production facility covering nearly 7 ha of land and producing 60,000 kg of product per day. Expansion plans, if completed, could double this amount to 120,000 kg per day. About 80% of the farm's output is destined for the domestic market while the remainder is exported to countries in Southeast Asia and Europe.

2.6 Outlook

China is the main producer and consumer of cultivated, edible mushrooms worldwide. Growth in the mushroom industry in China, especially since 1997, is an accomplishment seldom duplicated in agriculture today. China has become an enormous producer of cultivated mushrooms, accounting for about 87% of the world's total output.

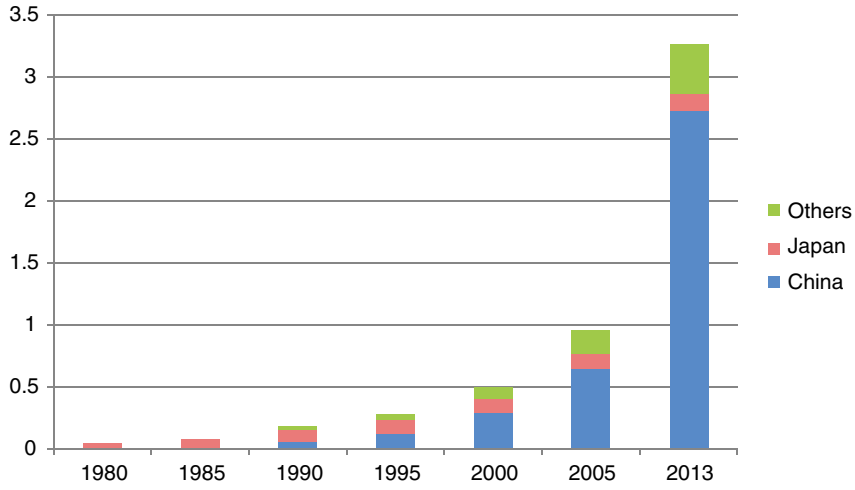


Figure 2.11 Growth in world production of *Flammulina velutipes* (billion kg) (1980–2013).

Lentinus edodes is now the world's leading cultivated edible mushroom with about 22% of the world's supply. Shiitake was traditionally cultivated on natural logs outdoors, but today most shiitake are cultivated indoors on nutrient-supplemented woodchips formed into varying shapes depending on the container in which they are grown. This allows for much faster production and leads to more crop cycles per year. *Lentinula* and four other genera (*Pleurotus*, *Auricularia*, *Agaricus*, and *Flammulina*) account for 85% of the world's total supply of cultivated edible mushrooms.

On average, consumers now enjoy about 5 kg of mushrooms per person per year. *Per capita* consumption is expected to continue to increase as consumers become more aware of the healthful benefits of incorporating mushrooms in their diet. Much more research is needed on the bioactive components in mushrooms to determine their biological responses in humans (Feeney *et al*. 2014). Promising evidence suggests that beta-glucan, vitamin D, selenium, and ergothioneine offer positive effects on immune function, intestine function, and weight management. It remains to be determined how often, how much and what species or mixtures of species should be consumed to bring about the desired biological response in humans. In the meantime, consumers can enjoy the unique culinary characteristics that mushrooms have to offer.

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3

Mushrooms: Biology and Life Cycle

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3.1 Life Cycle of Fungi

Mushrooms are macroscopic fruiting bodies produced by ascomycete and basidiomycete fungi during their sexual reproduction cycles. Most well-known species of consumed mushrooms belong to the group Basidiomycota. In addition, all cultivated species of economic importance are basidiomycetes, including such genera as *Agaricus*, *Pleurotus*, and *Lentinula*, which represent the majority of mushrooms cultivated in the world. Therefore, throughout this text, most of the information will be directed to basidiomycetes mushrooms; however, certain important ascomycetes will be addressed, including model species that do not produce mushrooms.

The life cycle of a fungus depends upon its nutritional strategy, which also defines the level of difficulty in cultivating the mushroom species for commercial purposes. Accordingly, fungi decomposers (saprophytes) are, in principle, relatively easy to cultivate. In contrast, mycorrhizal mushrooms, some species of which retain very high market value, are not able to be cultivated artificially, due to the need for direct interaction with specific tree species and complex interactions with other types of soil microorganisms, which until recently have been poorly understood. Of the many mycorrhizal mushrooms, truffle cultivation in artificial orchards has shown some success, but in general, the cultivation of certain *Tuber* species and other mycorrhizal mushrooms remains difficult.

Many species of fungi have the ability to modify their nutritional strategy to cope with environmental variation, or more often, the presence or absence of a host. Phytopathogenic fungi are a classic example of this versatility, as they switch from phytopathogenic to saprophytic modalities when their hosts die and are transformed into organic matter. This versatility enables them to survive until new living hosts with which to resume pathogenic activity become available. Mycorrhizal fungi can also exhibit saprophytic activity soon after germination of spores, until the hyphae establish a symbiotic relationship with the root tips of symbiont tree species.

In addition to varying nutritional strategies, the geographical origin of a particular species of mushroom can determine the specific change in temperature that induces its fruiting. In temperate climates, mushroom species are adapted to cycles imposed by well-defined seasons, making imperative the development of mechanisms for temperature recognition to trigger the beginning of a new cycle, as in the arrival of the autumn–winter period. It is common to refer to the lowering of temperature that induces fruiting as a stress factor. However, in nature, it is simply a mechanism that serves to define whether it is time to grow vegetatively, remain dor-

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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mant, or fruit. Moreover, it is important to emphasize that the temperatures most often used for fruiting induction (12–19°C) are considered mild temperatures in species acclimated to regions with severe winters, where the temperature frequently drops below zero. Under normal fruiting conditions, a temperature of 18°C would be interpreted by the physiological system of the fungus as time to fruit before winter arrives. However, for cold climate species, the same temperature would mean the end of winter and beginning of spring, a factor that triggers mushroom fruiting following a dormant period during the adverse conditions imposed by a severe winter.

In tropical countries where seasons are not well defined and temperature is maintained in a specific range, mushrooms may be produced throughout the year without requiring temperature-sensitive development triggers. The triggering factor for fruiting in these regions is rainfall, as fungi require a high relative humidity in order to fruit. It is therefore common to produce mycorrhizal mushrooms during summer months, when high temperatures are accompanied by periods of rain. Fruiting bodies normally occur after heavy rains, and drops of temperature during such periods can also play an important role in this process.

At first, it may seem to be advantageous to grow mushrooms that are not influenced by low temperature, especially in developing countries where the maintenance of temperature-controlled mushroom houses greatly increases production costs. In industrial-scale production, however, use of mushroom species adapted to concentrate their fruiting in narrower periods yields advantages associated with shorter crop cycles. *Agaricus bisporus* is the best-known example of this type of mushroom; its crop cycle concludes 30 days following the induction of fruiting. On the other hand, *Agaricus brasiliensis* (also known as *A. subrufescens* and *A. blazei*), native to Brazil and other warmer climates, does not require a temperature decrease for fruiting induction, but necessitates a long cultivation cycle of 2–3 months. Until now, the consensus has been that *A. brasiliensis* fruiting induction is not influenced by temperature variation, in accordance with its native environment of warm climate, where mushroom fruiting occurs in longer cycles and results in weaker flushes.

For mushrooms less amenable to cultivation, particularly those for which the induction of fruiting is not temperature dependent, it is important to study other factors implicated in triggering the fruiting process. As such, in this chapter we will discuss the general aspects of the life cycle of basidiomycetes, with an emphasis on sexual reproduction.

The cultivation of mushrooms presents as a basic prerogative the process of sexual reproduction during the fungal life cycle. Many species of fungi, most of them belonging to the ascomycetes group in which asexual reproduction is predominant, have no known sexual cycle. In addition, several species phylogenetically related to the basidiomycetes are classified as "*Mycelia sterilia*" as they are not known to produce spores. However, some of these species may reproduce sexually, but only under very specific conditions, making it difficult to induce sexual reproduction in the laboratory.

Most species that utilize sexual reproduction produce macroscopic fruiting bodies, which have been known to mankind since antiquity. Some of the most appreciated species for human consumption belong to the Ascomycota phylum; such mushrooms may be collected directly from nature or produced in artificial orchards. However, most of the mushroom species consumed by man, including several other species of wild mushroom as well as numerous cultivated species, belong to the Basidiomycota phylum. As a result, a large volume of research about the mechanisms involved in sexual reproduction is focused on the basidiomycetes. While there exists a great deal of information about the sexual reproduction of ascomycetes, these studies were done mostly with pathogenic species (particularly plant pathogens), species of biotechnological interest, or model species. In this context, much of the knowledge about fungal sexual reproduction is derived from studies of the ascomycete *Saccharomyces cerevisiae*.

This yeast has been a model species for many years apart from its great biotechnological importance, due to its easy cultivation and amenability to research. During the first half of the twentieth century, the filamentous ascomycete *Neurospora crassa* was an important model species for classical genetic studies. Subsequently, given their importance and the peculiarities that distinguish them from ascomycetes, the basidiomycetes have become objects of study as well, in order to better understand their mechanisms of sexual reproduction. Two of these species in particular, *Schizophyllum commune* and *Coprinopsis cinerea*, are now models for basidiomycete genetic research.

Although not the first to study the genetics of fungi, the great awakening in the genetics of basiodiomycetes occurred as a result of the work of John and Carlene Raper, summarized in *Genetics of Sexuality in Higher Fungi* (Raper 1966). *The Mycota*, edited by Karl Esser, also dedicated a significant component to fungal genetics. These scientists both contributed to the science of fungi directly and encouraged a new generation of fungal geneticists, among them Lorna Casselton and Ursula Kües, who went on to expand upon the legacy of John and Carlene Raper, both in the generation of scientific knowledge and in the formation of subsequent generations of fungal geneticists.

This chapter is not intended as a comprehensive review of the fungal life cycle, as there are already several exceptional works of deep detail for those interested in this field of science, for example, *Sex in Fungi: Molecular Determination and Evolutionary Implications* (Heitman et al., 2007), in addition to *The Mycota I* (Kües and Fischer, 2006). The purpose of this chapter is to present an introduction to the subject for the student or mushroom grower who is interested in entering the fascinating area of Mycology, enabling access to the basic principles of the mushroom life cycle with an emphasis on sexual reproduction and its underlying mechanisms.

3.2 The Subkingdom Dykaria

Ascomycetes and basidiomycetes are unique in the kingdom Fungi for the dikaryotic phase of their life cycle. Due to this feature, these two phyla exclusively form the subkingdom Dikarya, and are known as higher fungi as a result of their greater complexity and ability to produce macroscopic structures. The two groups are distinguished from one another by their modes of sexual spore production (ascospores versus basidiospores), from which the names of the two phyla were derived. Ascomycetes and Basidiomycetes have other, minor differences, as well, such as dikaryophase time and mating type genes.

Dikaryosis is generally understood as an evolutionary stage between haploidy and diploidy; it remains intriguing how these evolutionarily arrested organisms have maintained this condition without losing their competitiveness. In fact, evidence affords a competitive advantage to the dikaryon under heterogeneous environments, as the dikaryon is more flexible, with its greater phenotypic amplitude with which to cope with environmental variations. While certain species of Basidiomycetes form a diploid rather than undergoing a dikaryophase following plasmogamy (e.g., *Armillaria mellea*), the vast majority of known species of basidiomycetes spend most of their life cycle in dikaryophase. Because of this, it is important to understand the different characteristics of the dikaryophase of ascomycetes versus basidiomycetes.

3.2.1 Dikaryosis: Concepts

Dikaryosis is defined as an association of haploid gametic nuclei in a single compartment that is not immediately followed by karyogamy. This is a unique phenomenon found only in fungi; most eukaryotes undergo plasmogamy and karyogamy in rapid succession, yielding a diploid

nucleus. For ascomycetes and basidiomycetes, however, plasmogamy and karyogamy are temporally disparate events, in particular for basidiomycetes, in which the dikaryon remains for an extended period following plasmogamy. For these organisms, the dikaryophase comprises a major period of the fungus's life.

In a typical dikaryon, two haploid nuclei are paired in the same compartment and maintain their individual haploid status rather than fusing into a single, diploid nucleus. *Schizophyllum commune*, *Coprinopsis cinerea*, and *Lentinula edodes* are examples of species that produce dikaryons. Some species of basidiomycetes have multinucleated compartments, and are not dikaryons in the strict sense of the word; nonetheless, current use of the term dikaryosis is not restricted to situations wherein only two haploid nuclei occupy the compartment. Additionally, many authors refer to an individual with two types of gametic haploid nuclei derived from different parents as a dikaryon, and the term dikaryon is also used synonymously with heterokaryon, wherein different nuclei are present in the same compartment. *Agaricus bisporus* and *A. subrufescens* are examples of basidiomycetes that produce heterokaryons with multinucleated compartments. Such nuclear behavior differences consequently result in morphological differences, which will be discussed later.

While both are dikaryotic, Ascomycota and Basidiomycota display some key differences. As mentioned previously, for basidiomycetes, the dikaryophase is a prolonged, vegetative stage, whereas for ascomycetes, the dikaryon is usually restricted to the ascogenic system within fruiting bodies, especially when the partner that acts as female produces a morphologically distinct structure to fulfill this role. In this context, it is important to emphasize that ascomycetes display morphological differences between male and female partners, whereas basidiomycete partners are usually morphologically indistinguishable. Another important morphologic difference is that the basidiomycete produces clamp connection at each apical cell division, whereas ascomycetes produce an alternate structure called a crozier. The clamp connection in basidiomycetes maintain an ordered heterokaryotic state of hyphae at every cell division. The crozier ensures dikaryosis in the ascogenic hyphae, where the ascus will be produced, in a similar function to the clamp connection, but to a more limited effect. While a clamp connection appears immediately after the dikaryon is established, a crozier is produced later in the ascogenic hyphae. Despite these morphological variations, ascomycetes and basidiomycetes share the characteristic of presenting an intrinsic combination of two haploid nuclei originating from the genotypes involved.

This leaves the question of how dikaryons are formed. In the following sections, we will discuss the basic mechanisms that control breeding between basidiomycetes and the structures and external factors involved in the process.

3.3 Homothallism, Heterothallism, and Amphithallism

First, it is important to become familiarized with the different types of hyphae, from germinating spores to the formation of a fruiting body. Germination of a uninucleate sexual spore or binucleate with identical nuclei gives rise to monokaryotic and homokaryotic hyphae, respectively.

Interaction between hyphae from different thalli leading to plasmogamy followed by nuclear migration to the tip of the hypha results in a complete dikaryotic mycelium known as dikaryon. Since cell fusion occurs between different, compatible individuals (with different nuclei), and the resulting dikaryon will also be a heterokaryon.

For homobasidiomycetes, there is a principle of differentiation during the formation of fruiting bodies that leads to the formation of a pseudotissue known as the pseudoparenchyma. In simpler language, different types of hyphae may be referred to as the primary mycelium (monokaryon), secondary mycelium (dikaryon), or tertiary mycelium (pseudoparenchyma). These terms are rarely used today, particularly in the context of genetics and molecular biology; however, they remain practical for a less specialized audience.

The monokaryon and dikaryon are less differentiated than the pseudoparenchyma (fruiting bodies), but it is possible to observe morphological differences between them. Typically, the monokaryon displays less robust mycelial growth compared to the dikaryon, and the loss of monokaryotic cultures following several subculture cycles is not uncommon, in function of its weakness of growth. The dikaryon, in turn, has more vigorous growth and is much more stable. In addition, for many species of basidiomycetes, the dikaryon produces a structure known as the clamp connection. This clamp connection allows each compartment to receive two distinct nuclei, maintaining the heterokaryotic state of the hyphae. The clamp connection is therefore an important morphological marker that distinguishes the dikaryon from the monokaryon in such species. However, this is only possible for the basidiomycetes that produce typical dikaryons. In *Lentinula edodes*, for example, the dikaryon produces frequent clamp connection that are easily observable under optical microscopy; this is not the case for *Agaricus bisporus* and *A. brasiliensis*. For these two species, a fertility test of monosporic cultures or the use of molecular markers is necessary to distinguish between homokaryosis or heterokaryosis (Nazrul and Yin Bing, 2011; Rocha de Brito et al., 2016).

3.4 Heterothallism

A fungal species is considered heterothallic when its sexual spores germinate autosterile monosporic cultures thereby requiring a cross with another culture (thallus) to generate a dikaryon, which is then able to complete the life cycle. In this case, the sexual spores have a single nucleus, or when the spores are binucleate, the nuclei are identical. *Lentinula edodes* is an example of a heterothallic species, as their monosporic cultures are unable to produce fruiting bodies unless they are crossed with another compatible monosporic culture.

3.5 Homothallism

A fungus is considered homothallic when a colony originating from a single spore is able to complete its life cycle, producing fruiting bodies via autofertilization. A homothallic species allows inbreeding among genetically identical hyphae and sharing of identical nuclei in the same compartment. However, certain homothallic species nonetheless require different genetic factors to consolidate the sexual cycle, present in different nuclei. Therefore, although specific sexual factors are required, these individuals are considered homothallic, since their monosporic cultures are self-fertile. It is important to emphasize that the concepts of homothallism and heterothallism were established in the context of whether a single thallus was able to undergo a complete life cycle without consideration of the necessity of distinct mating types genes.

To permit distinction between these species and typical homothallic species, the former is referred to as "secondary homothallic" or "pseudo homothallic," that is, they fulfill the basic requirements for homothallism; however, the presence of two distinct sex type genes in the same compartment is required for completion of the sexual cycle.

The typical species example of this system is the button mushroom *Agaricus bisporus*, known to produce predominantly binucleate spores with sexually distinct nuclei, able to produce fruiting bodies without being crossed with another culture. The categorization is imperfect, however, as the same species may differ in the number of spores per basidia. In the case of *A. bisporus*, a small percentage of basidia produce four, rather than two, basidiospores. These spores do not give rise to self-fertile cultures and are therefore considered heterothallic. Adding to this complexity, a particular *A. bisporus* strain has been found that predominantly produces four spores instead of the typical two spores per basidium (Callac et al., 1993). Therefore, even within the same species, both secondary homothallic and heterothallic strains can exist.

3.6 Amphithallism

Further complicating matters are the amphithallic species, able to produce both homokaryotic and heterokaryotic spores, such that the same strain may give rise to both secondary homothallic and heterothallic cultures. The most common (but not only) circumstances in which this occurs are when bisporic and/or trisporic basidia are present alongside tetrasporic basidia. Bisporic and trisporic basidia give rise to binucleate spores, usually with sexually distinct nuclei, while tetrasporic basidia normally give rise only to homokaryotic spores. Therefore, the same basidiocarp is able to produce spores that will follow a heterothallic life cycle (homokaryotic spores) as well as spores that follow a secondary homothallic life cycle (heterokaryotic spores), a condition defined as amphithallism. The *A. brasiliensis* species is a textbook example of this type of life cycle, displaying wide variation in its production of bisporic, trisporic, and tetrasporic basidia (Kerrigan, 2005). This feature can be influenced by environmental conditions, mainly temperature, but studies with *A. brasiliensis* also show that different strains produce different ratios of tetrasporic, trisporic, and bisporic basidia even when cultured under the same environmental conditions (Herreira et al., 2012).

While genetic determinants are somewhat responsible for these production ratios, other factors can also facilitate the transition between heterothallism and homothallism within the same species, particularly in the ascomycetes group. The historic research model yeast *S. cerevisiae*, for example, has both heterothallic and homothallic strains. Those that are homothallic have a heterothallic control mechanism; as the cells divide, a switching mechanism promotes the replacement of one mating type for the other. This mechanism allows the changing of the mother cells to the opposite mating type, thus ensuring the presence of cells of different mating types in a previously autosterile culture. According to this model, pseudo homothallism is a strategy to break down heterothallic genetic control. Besides mating type switching and the production of heterokaryotic spores, other mechanisms of homothallism include the presence of unlinked or occasionally fused mating type loci. These mechanisms produce the same results as found in heterokaryotic spores, but with the different mating types present in a single nucleus. For a better understanding of this wide range of mechanisms, it is necessary to delve into the genetic underpinnings of these processes; we recommend the book *Sex in Fungi: Molecular Determination and Evolutionary Implications* (Heitman et al., 2007) as an excellent resource.

As discussed earlier, the concepts of homothallism and heterothallism were originally defined in the context of self-fertility versus self-sterility of monosporic fungal cultures prior to knowledge of the genetic factors responsible for these different phenotypes. Research has since shown that such characteristics are controlled by an intricate genetic control interplay, the purpose of which is to trigger a series of biochemical responses via a pheromone system of

ligand–receptor interactions on the cell membrane that vary among species. However, certain main genes and their products will be discussed next.

3.7 Mating-Type Genes

For any eukaryotic organism, sexual reproduction is important to produce descendants, and for the generation of genetic variability. For fungi, in which morphological distinctions between male and female individuals are not always apparent, it is necessary to have mating-type genes to ensure that such interactions can occur. The genes involved in the process include those whose function it is to prevent self-crossing in order to promote genetic variability.

Taking, for example, a heterothallic species, the cross between two individuals (thalli) will occur only if the two parents are sexually compatible, or of distinct mating type genetic complements. Two mating-type systems can be found in these species. In the first, known as a bipolar system, one locus ensures the compatibility of the interaction, such that two individuals are compatible if they display alternate alleles at this locus, most often defined as **a** and **α**, MATa and MATα, or EHV-1 and EHV-2. The second, a tetrapolar system, involves two unlinked loci behaving as independent genes. This system generates spores of four different possible mating types, and to have compatibility between thalli, it is necessary that the two individuals be different at both loci. For example, an *A1B1* individual will be compatible with another individual *A2B2*, but will not be compatible with an *A2B1* individual, although some exceptions have been observed. It is interesting to note that a large number of alleles can be found for the two loci, so that the probability of finding a compatible individual is very high, making the outcrossing probability almost 100%.

Considering the distribution of the two systems between ascomycetes and basidiomycetes, there is a preference by ascomycetes for the bipolar system, while the tetrapolar system is somewhat more common in basidiomycetes, although a significant number of basidiomycetes favor the bipolar system. Homothallism is more common among ascomycetes, but among basidiomycetes, the Agaricomycetes, a mushroom-producing group, is mostly heterothallic. Of importance, a large number of secondary homothallic species have evolved from the heterothallic and bipolar system. A plausible cause for this is that it is easier to bypass heterothallic control and generate secondary homothallism in a system controlled by a single locus.

As mentioned previously, the yeast *S. cerevisiae* has become the primary model for detailed studies on mating type genes. Based on discoveries made with *S. cerevisiae*, these same mechanisms were found in filamentous ascomycetes and basidiomycetes, including the Agaricomycetes. It is now well established that although there are many variations, they are all derived from a single system. In brief, one locus is responsible for coding pheromones and pheromone receptors, while another locus encodes transcription factors that regulate gene expression along a sequence of events resulting in the migration of the nucleus to the apical hyphal compartment, formation of the dikaryon, and finally karyogamy and meiosis processes that generate basidiospores and ascospores.

Among the many variations, for *S. cerevisiae* and for several filamentous ascomycetes species, the pheromone–receptor interaction promotes attraction and cell fusion; however, for mushroom-forming basidiomycetes cell fusion can occur independently of this interaction. Rather, that system is utilized to facilitate other important processes, such as the formation of the clamp connection.

In *C. cinereus*, for example, the interaction between hyphae does not require pheromone–receptor contact. Only after the hyphal fusion has taken place and both nuclei are paired do the mating type genes determine the sequence of events. This explains the formation of a mycelial network by anastomosis in the absence of sexual reproduction. This does not mean that other

genetic factors are not necessary to ensure vegetative compatibility for anastomosis. In fact, such mechanisms are well described for the species of ascomycetes.

Mating type genes are located at two, unlinked loci (located at different linkage groups in the genome). These genes are known as *A* and *B*, and it is necessary that crossing partners bear different alleles at each of the loci for the sexual process to occur. In the case of basidiomycetes, these genes are multi-allelic (represented by a large number of alleles), which greatly increases the probability of outcrossing.

The *B* genes encode pheromones and pheromone receptors which are not required for cell recognition, as with *S. cerevisiae*, but rather trigger the nuclear migration process and facilitate formation of the clamp connection, when the hook must merge with the hyphal compartment that will receive the nucleus. In this aspect, the pheromone–receptor system plays a similar role to that observed in *S. cerevisiae*, but far later in the process and with a completely different purpose, a remarkable variation of the system.

In turn, the *A* genes encode transcription factors that enable the expression of genes required for synchronization between the division of the nucleus and the formation of the clamp connection. Functional transcription factors consist of two monomers, each from the different mating type partners, resulting in a heterodimer. In the same way that a receptor does not recognize a pheromone from its own mating type, a protein monomer does not form a functional transcription factor with a monomer derived from the same mating type. Therefore, the presence of different alleles for both genes (*A* and *B*) is necessary for the development of the dikaryon and the successive events that will culminate in meiosis and the production of sexual spores.

Certain rare mutations can disrupt regulation of this process. For example, a mutation that results in nonselective pheromone recognition by a receptor can override the requirement for distinct mating types. Likewise, changing one or two amino acids in a protein encoded by an *A* gene may allow the formation of heterodimer from monomers originally designated incompatible, underlining the sensitivity of the recognition mechanism, wherein small changes in the genome can result in drastic changes in pairing compatibility. Even for the basidiomycetes, small genetic changes can result in significant morphological differences among fruiting bodies. This often resulted in mushroom species misidentification, back when the taxonomic standards were restricted to the morphological characteristics of fruiting bodies and sexual spores.

3.8 *Agaricus brasiliensis* (Syn = *A. subrufescens* or *A. blazei*): An Intriguing Example of Amphithallism

Hundreds of papers have been published about this mushroom, but only a small number refer to cytology, nuclear behavior, or the fungal life cycle. The poverty of work in this area undoubtedly reflects the difficulty in studying a species that does not have the classic dikaryotic system. The first work on the cytology of *Agaricus brasiliensis* showed that the hyphae of heterokaryotic cultures were multinucleated (Labory et al., 2003). The presence of multinucleated hyphae was evidence of a more complex life cycle as compared to typical dikaryons. In a later study (Dias et al., 2008) with different strains, it was evident that the number of nuclei can range from 1 to 15, wherein the most frequent number was five per compartment, followed by six and four, respectively. An odd number of nuclei indicates that one of the nuclei has not divided and should be considered to be in a transitional phase; as such, it was concluded that *A. brasiliensis* most frequently have six nuclei per compartment. In this same study, the size of the compartments was determined as well as the diameter of the hyphae in different strains. Diameters

ranged from 3.5 to 7.0 μm with most between 4.0 and 5.0 μm, and compartment length was generally between 50 and 100 μm.

One of the most interesting aspects of the work was the fluorescence microscopy of nuclear behavior during the formation of basidiospores in a strain that produces tetrasporic basidia. The authors have chosen this strain for the study of nuclear behavior as basidiospores overwhelmingly produce the same number of nuclei. Using this approach, we observed spores containing only a single large nucleus and spores containing two smaller nuclei, suggesting that each basidiospore should present only one meiotic nucleus followed by post-meiotic mitotic division giving rise to two homokaryotic nuclei. Given these data, it was suggested that this species follows a principle of heterothallism, as homokaryotic monosporic cultures are self-sterile.

However, Herreira et al. (2012) later demonstrated that this species may vary in the number of basidiospores by basidia depending on the strain (Figure 3.1). According to Herreira, the frequency of tetrasporic basidia can be as low as 29.9% in the strain CS7, while the frequency of trisporic basidia can be 46.9% and that of bisporic basidia 23.2% in the same strain. These

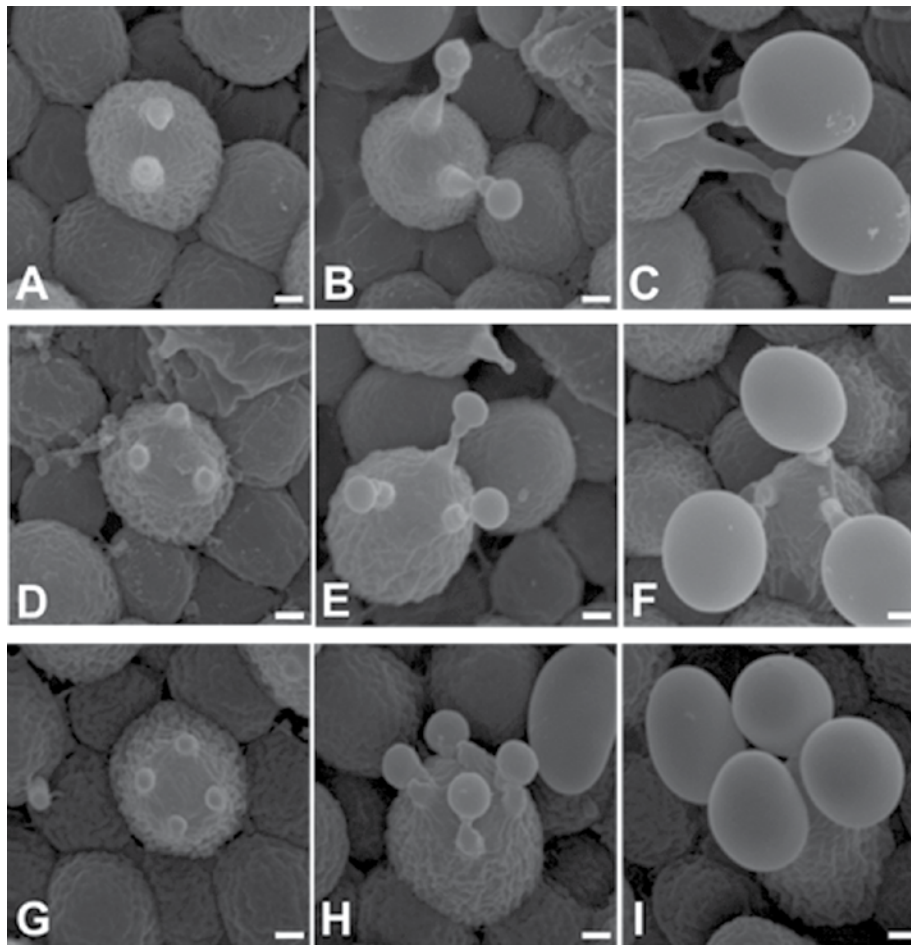

Figure 3.1 Scanning electron micrographs of *Agaricus brasiliensis* gills. A–C: Bisporic basidia. D–F: Trisporic basidia. G–1: Tetrasporic basidia. Bar = 1 mm. Herreira et al. (2012), Mycologia. With permission.

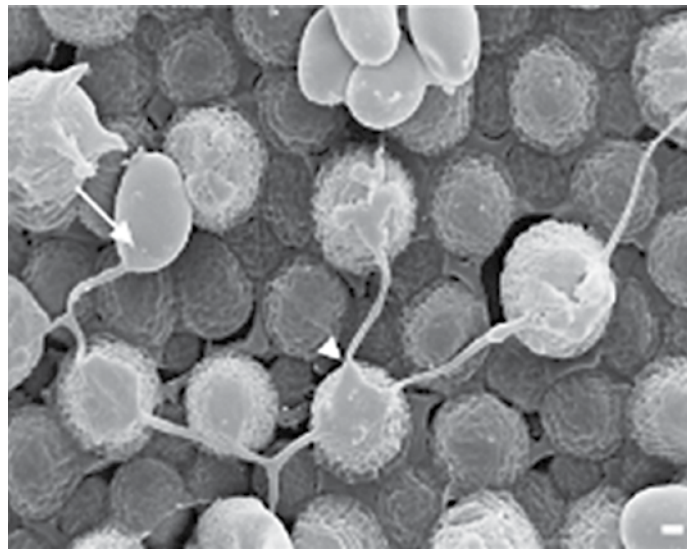

Figure 3.2 Scanning electron micrographs of *Agaricus brasiliensis* gills. The arrow indicates a basidiospore linked to a connection hyphae and two basidia. Arrowheads indicate sterigmata connected to one another by a hypha. Bar = 1 mm.

values are quite different from strain CS10, which presented frequencies of 93.9, 5.9 and 0.2% of tetrasporic, trisporic, and bisporic basidia, respectively. These results showed that large numbers of basidiospores containing two distinct nuclei will generate self-fertile, monosporic cultures, indicating a shift to secondary homothallism. These results confirm an earlier statement by Kerrigan (2005) that this species is actually amphithallic.

An intriguing finding in the work of Herreira was the production of connection hyphae between basidia of *A. brasiliensis* (Figure 3.2). At first, it was suggested that these structures allowed for the passage of nuclei from one basidia to another; however, Ribeiro (personal communication) later reported that the diameter of connection hyphae is too narrow to allow for nuclear migration. As such, the function of these structures remains a mystery.

Returning to the question of amphithallism, Thongklang et al. (2014) uncovered a high heterokaryon rate (40.43–75.46%, depending on the strain). The authors did not detect bisporic basidia, and trisporic basidia were rare (less than 1%) between basidia from the three parental strains used in the work. Considering that the basidia produced by each strain are predominantly tetrasporic, the high frequency of amphithallism in these strains can not be explained by the production of binucleate basidiospores derived from trisporic or bisporic basidia alone. These results instead suggest that heterokaryotic spores are formed due to post-meiotic mitotic division inside the basidia rather than the basidiospore. Further studies using eletron microscopy may be necessary to clarify nuclear behavior during formation of basidia and basidiospores.

3.9 Life Cycle of Uncultivated Mushrooms

Some of the most appreciated edible mushrooms in the world are produced from mycorrhizal fungi, particularly from symbiotic associations with trees from the genera *Pinus*, *Quercus*, and *Corylus*. Of all known species of mycorrhizal mushrooms, we have chosen to highlight *Tuber*

and *Morchella* (ascomycetes), and *Tricholoma*, *Boletus,* and *Cantharellus* (basidiomycetes). All of these genera have in common a much more complex life cycle as compared to the saprophytic mushrooms. This renders their cultivation far more difficult, and their consumption has been possible primarily due to their frequent natural occurrence during autumn, winter, and spring, depending upon species and geographical region. In recent decades, great efforts have been undertaken to develop cultivation techniques for these mushrooms in association with their symbiont plants (Hall et al., 2003). Some of these are discussed next.

3.10 The Truffles

The genus *Tuber* (Ascomycota) encompasses several species of which we highlight *T. melanosporum*, known as black truffle or black Périgord, and *T. magnatum*, known as Italian white truffle. For a long time, these mushrooms were predominantly European in origin, as a result of their natural occurrence and traditional continental consumption. However, due to the decline in the wild truffle harvests over the years, European countries and others with favorable climates have stepped up efforts to cultivate these mushrooms in commercial orchards. Oak (*Quercus* spp.) and hazelnut (*Corylus avellana*) are preferred species for commercial crops. However, proper handling is necessary to minimize competition from other mycorrhizal species in the establishment of the crop, since species of interest are not always robust in effecting a mycorrhization process, as has been observed with *T. magnatum*.

The life cycle of these mushrooms is not as well known as that of saprophytic mushrooms, as it is still not possible to carry out all stages of their life cycle in a laboratory setting. However, it is believed that truffles follow the same basic pattern as other species of ascomycetes, with particular differences, such as those pertaining to interactions with other soil microorganisms. The life cycle begins with the germination of spores which originate homokaryotic hyphae able to interact with the roots of the host plant. Hosts can harbor primary hyphae of different mating types, and these hyphae can extend up from the mycorrhizae growing in the soil to meet hyphae of the opposite mating type, resulting in plasmogamy and giving rise to the ascogenic hyphae, which then undergo subsequent processes of karyogamy and meiosis. As also occurs in other ascomycetes, the ascocarp is not constituted, uniformly, by heterokaryotic hyphae, since plasmogamy usually occurs within the structure which will originate the ascocarp. Following plasmogamy, the ascogenic hyphae is surrounded by homokaryotic hyphae of maternal origin, which are responsible for the formation of the majority of the truffle. Some evidence indicates that plasmogamy occurs only after the primordium is formed, such that the heterokaryotic phase of the fungus is extremely brief.

Truffles are usually harvested during autumn and winter. Depending on the region, the harvest may begin as soon as temperatures start to drop in the fall, or earlier according to the timing of rainy periods. Harvests can end before winter or shortly after it begins. In other regions, harvest may begin later in the fall and continue on through winter. In general, the sexual cycle is stimulated by a temperature drop the following summer, and spores survive through the winter and then restart the cycle in the spring.

3.11 Morels

The *Morchella* genus is responsible for the production of mushrooms known as morels, among which the species *M. esculenta* stands out. Morels were originally considered saprophytic, but now it is believed that this species can also present as mycorrhizal. Although

they are a cosmopolitan species found in various regions and continents, these mushrooms are strongly identified with North America, where a tradition of professional collectors has sprung up to meet market demand. Although they are not mycorrhizal-obligate fungi (Tedersoo et al., 2013) and can live as saprophytes, cultivation as such is extremely difficult. This species has adapted to a fire ecology, since naturally occurring, low-intensity fires are key to increasing mushroom production in the wild (Wurtz et al., 2005). Without such events, mushrooms are produced in much lower numbers. The fire effect generally manifests one or two summers following the fire. Their life cycle is marked by the production of sclerotia, which are resistant structures usually formed by the secondary mycelium. After a disturbance event, the sclerotia germinate to form an ascogenic mycelium, which then gives rise to the fruiting body (morel). Several environmental factors (extreme temperatures, heavy rainfall, lack of humidity, prolonged winter, etc.) can interrupt the dormancy of sclerotia, but as previously mentioned, fire appears to be the most crucial. Production of sclerotia is therefore regarded as an essential step in the life cycle of these fungi, without which fruiting bodies are not intensely produced.

3.12 The Chanterelles

The *Cantharellus* genus houses species of mushrooms known as chanterelles, which are also identified with North America (Pilz et al., 2003). These mushrooms also have a long tradition of consumption in Europe, but their occurrence has declined in recent decades, as has been observed with other species. *C. cibarius* produces the mushroom known as golden chanterelle, as ranges in color from yellow to orange. In North America, *C. formosus*, also known as the "Pacific Golden Chanterelle" is the best-known species. Other genera such as *Craterellus*, *Gomphus*, and *Polyozellus* also produce mushrooms referred to as chanterelles; however, only species in the *Cantharellus* genus are considered "true" chanterelles. Within this genus, there are many known species that are associated with different species of plants such as spruces (*Picea*), pine (*Pinus*), Douglas fir (*Pseudotsuga*), and oak (*Quercus*). As occurs with morels, chanterelles also support a tradition of professional collectors in North America; however, most of the mushrooms collected are exported to the European market.

Unlike other mycorrhizal mushrooms, which are typical of temperate regions in the Northern hemisphere, several species of chanterelles are also found in tropical countries, including Africa, Asia, and Latin America. This wide distribution indicates a greater versatility of this mushroom with respect to the need for low temperatures for fruiting induction. Periods of summer and hot springs, with proper humidity, favor the production of this mushroom even in the more temperate regions of North America. This adaptation to different environmental conditions seems to have enabled its wide geographic distribution.

Cantharellus has a life cycle even more complex than the other mycorrhizal mushrooms, because in addition to the mycorrhizal association, the fungus closely associates with other organisms that grow in its tissues (Garbaye, 1994), making its cultivation even more difficult. Isolation and identification of these microorganisms would vastly accelerate the development of cultivation technology. Unfortunately, attempts thus far have not been successful enough to enable cultivation on a commercial scale. Reproduction of the conditions that occur in nature, especially interactions with other microorganisms, including some uncultivable microorganisms, remains a considerable obstacle.

3.13 The Matsutake

Tricholoma matsutake or simply matsutake is a native mushroom in Japan, strongly identified with this country, and occupies the same position as truffles occupy in Europe. Also, just as for truffles in Europe, this mushroom has faced a huge decline in natural production output, and attempts to develop techniques for commercial production have yet to be successful. In North America, another species (*T. magnivelare*), known as American matsutake, can still be found in abundance, allowing for its commercial exploitation to meet the demand of both the American and Japanese markets.

The name of this mushroom originates from its host tree species ("matsu" for *Pinus* and "take" for mushroom) and can be associated with different pine species and other arboreal genera such as *Quercus*, *Castanopsis*, and *Picea*, depending upon geographical region and *Tricholoma* species. Like other species of mycorrhizal mushrooms addressed in this text, the matsutake is a typical Northern hemisphere mushroom, whose natural fruiting occurs between late summer and autumn, when temperatures drop. The production of this mushroom is associated with a structure known as the "shiro," which is defined as a dense mycelial mass that aggregates along roots and in soil particles (Guerin-Laguette et al., 2003; Vaario et al., 2011). However, while it is the dominant microorganism, *Tricholoma* hyphae are not alone in these structures, as a large variety of prokaryotes and other fungi are also present. It has been surmised that at the time of fruiting, *Tricholoma* use an alternative energy strategy involving the degradation of organic compounds, particularly hemicellulose. In this context, the different members of the Shiro microbial community likely play an important role in the production of the necessary enzymes. Such factors underline the complexity of the life cycle of this important species of mushroom as well as the difficulties in its cultivation, since it remains prohibitively challenging to reproduce the precise combination of environmental factors found in nature that culminate in a succesful sexual cycle of the fungus.

3.14 Porcini

Mushrooms known as porcini or King Boletus are a group of species from the *Boletus edulis* sensu lato. Species distinction among *B. aestivalis*, *B.aereus*, and *B. reticulatus* is only reliably performed by specialists, and for commercial purposes, all of them are referred to as belonging to the *B. edulis* complex. All these species are mycorrhizal, associating with different families of tree species, such as *Fagus*, *Pinus*, *Picea*, *Quercus*, and many others, depending on the region or species preference of the fungus itself. This group of mushrooms in also native to the Northern hemisphere, although the species *B. loyo* can be found naturally in Chile (Deschamps, 2002). *B. edulis* is now also found in certain Southern hemisphere countries such as Australia, New Zealand, and South Africa, and is believed to have been brought with its preferred tree species as they were imported from Europe (Hall et al., 1998).

As observed for morels, chanterelles, and matsutake, attempts to establish tree orchards inoculated with this fungus have been unsuccessful. Studies with ectomycorrhizae have shown that the microorganisms present in the mycorrhizal mantle, in particular the bacteria, play a vital role in the process; this has also proven to be the case with *B. edulis* (Wu et al., 2012). Other studies indicate that basidiocarp production is affected by factors outside of the mycorrhizal association. The balance of all the factors the fungus has become adapted to as it has evolved in its native environment is quite delicate and extremely difficult to reproduce. Establishment of orchards containing just one species of host tree for the fungus does not allow for the presence

of other plant species that may be essential for the establishment of the proper microbiota required to support all interactions that will culminate in the production of mushrooms.

3.15 Decreased Production of Mycorrhizal Mushrooms in the Northern Hemisphere

Strong evidence of the decline in production of mycorrhizal mushrooms in the Northern hemisphere has been well established. At first, it was speculated that this decline resulted from human intervention, since commercial interest has led to an intense exploitation of the areas of natural production of these mushrooms. Several other aspects were considered, as well, including soil compaction and environmental disruption either leading to early abortion or otherwise compromising the formation of mature fruiting bodies. These studies have shown that the answer cannot be found in any single factor; other factors besides anthropogenic action are implicated in this decline.

However, even if we wished to ascribe all of the problems observed in nature to man, we must recognize that we live in a changing world. Although anthropogenic action has contributed greatly, it alone cannot explain recent climate changes and mass extinctions of species. For example, when the Sahara region changed from a lush to an arid region, human technology was far from the power of destruction it is today. Climate change in the region nonetheless upset its existing balance through a shift in rain cycles, and only a few species were able to survive; the rest were replaced by others better adapted to the new environment.

It is undeniable that the climate is changing, no matter the cause. While the hottest regions will suffer the most obvious consequences of global warming, even in temperate regions, subtle elevations in temperature can have important ecological consequences, having a significant impact on life cycles of native species. The soil itself, with its intricate complexity of interactions between plants, animals, and microorganisms, can be transformed in response to environmental changes.

The assessment of changes in different ecosystems is relatively easy considering the animal and plant species that grow on its surface. However, drastic changes in the soil of an ecosystem have often begun long before their effects become visible. Therefore, it is important to consider the possibility that a decline in the production of mycorrhizal mushrooms is just a sign of an ongoing transformation process that has yet to reach its greatest amplitude.

It is possible that the mushroom production decline represents a fitness cost, as resources are diverted so that the organism can adapt to changes in its environment. The sexual cycle is an energetically expensive process, and the need to invest in mycelial growth may result in a negative correlation to sexual reproduction. Quantifying the production of fruiting bodies as an evaluation criterion of fungal fitness may be an important tool for the study of different species of mycorrhizal mushrooms. In this context, the study of non-edible species could yield important information, as such species are not subject to intense pressures that result from the harvest of edible species. Comparisons of fitness between edible and non-edible species could greatly enhance our understanding of the decline in the production of edible mycorrhizal mushrooms.

3.16 Fitness of Filamentous Fungi

Fitness can be defined as an organism's ability to respond to situations that necessitate increased energy expenditure, such as distributing oxygen to muscle tissues. In this report, the concept of

Darwinian fitness will be used, of which the simplest definition is "the ability of a population to maintain or increase their number in succeeding generations" (www.dictionary.com/browse/darwinian-fitness). According to Antonovics and Alexander (1989), biological fitness can be defined in the context of individual selection as "the contribution of a phenotype to a subsequent generation." According to the authors, it is more appropriate to define fitness in phenotypic rather than genotypic terms, especially when dealing with quantitative traits (those determined by several loci). One of the main reasons for this is that the measurement of a phenotype is easier and more objective than the measurement of a genotype. While we can measure changes in the copy number of a gene from one generation to the next, fitness comprises not only copy number, but also the gene expression influenced by environment. Finally, Pringle and Taylor (2002) defined fitness as a function of "survival and reproductive success of an allele, individual, or group."

For each filamentous fungus group, the fitness will be influenced by its nutritional strategy: saprophytism, simbiosis, or parasitism. For edible and/or medicinal mushrooms, the first two strategies are of primary interest, because the great majority of such mushrooms are produced by saprophytic or mycorrhizal fungi. However, most fitness studies up to this point have been conducted on species of pathogenic and saprophytic fungi that attack seeds or post-harvest fruit, in accordance with their economic importance (Pringle and Taylor, 2002). One exception was Xu's (1995) work with *Agaricus bisporus*, which is a cultivated species of basidiomycete. When evaluating different aspects of fitness of this species, Xu reported inbreeding depression in *A. bisporus* resulting in a decrease in fitness due to the loss of genetic variety. According to the author, inbreeding depression is a phenomenon already observed in other species of basidiomycetes such as *Pleurotus sajor-caju* and *Volvariella volvacea*. Some of the most important species of mycorrhizal mushrooms are basidiomycetes, such as *Tricholoma matsutake* and *Boletus edulis*. Could these species also be suffering from inbreeding depression?

The basidiomycetes, and in particular the Agaricomycetes, are characterized by promiscuous sexual behavior, with an increased number of mating type genes theoretically ensuring a high rate of outcrossing (James, 2015). However, while millions of spores are released by each basidiocarp, few of them will be dispersed over long distances. Therefore, there is a high probability of crosses between individuals resulting from the same meiotic event, as a multitude of these spores remain but a small distance from the basidiocarp. Consequences as to the fitness of these mushroom species under such conditions remain unclear. For basidiomycetes, sexual reproduction is an obligate step in the life cycle of these fungi, although studies of possible mechanisms of asexual reproduction have not been properly considered for this group. While ascomycetes, with their large numbers of sexual homothallic species, have well-characterized asexual reproduction modalities, the sexual cycle seems to occur regularly during the life cycle of mycorrhizal species. Conversely, for ascomycete and basidiomycete mycorrhizal mushrooms, the sexual cycle is not well clarified, although research is ongoing. Volk and Leonard (1990) proposed a basic life cycle for the fungus *Morchella*. Subsequently, Alvarado-Castillo et al. (2014) have expanded on the details of this cycle, however, with a number of as yet unproven possible routes (Figure 3.3). For species of the *Tuber* genus, there have also been recent entries with regards to life cycle (Paolocci et al, 2006; Kües and Martin, 2011) and much effort is being invested in the study of these mushrooms, sometimes resulting in relative success in the establishment of commercial orchards. Nevertheless, the environmental complexity remains a major challenge, and there is much remaining work to be done in delineating the set (or sets) of factors that are required for optimal fitness and cultivation of mycorrhizal mushrooms.

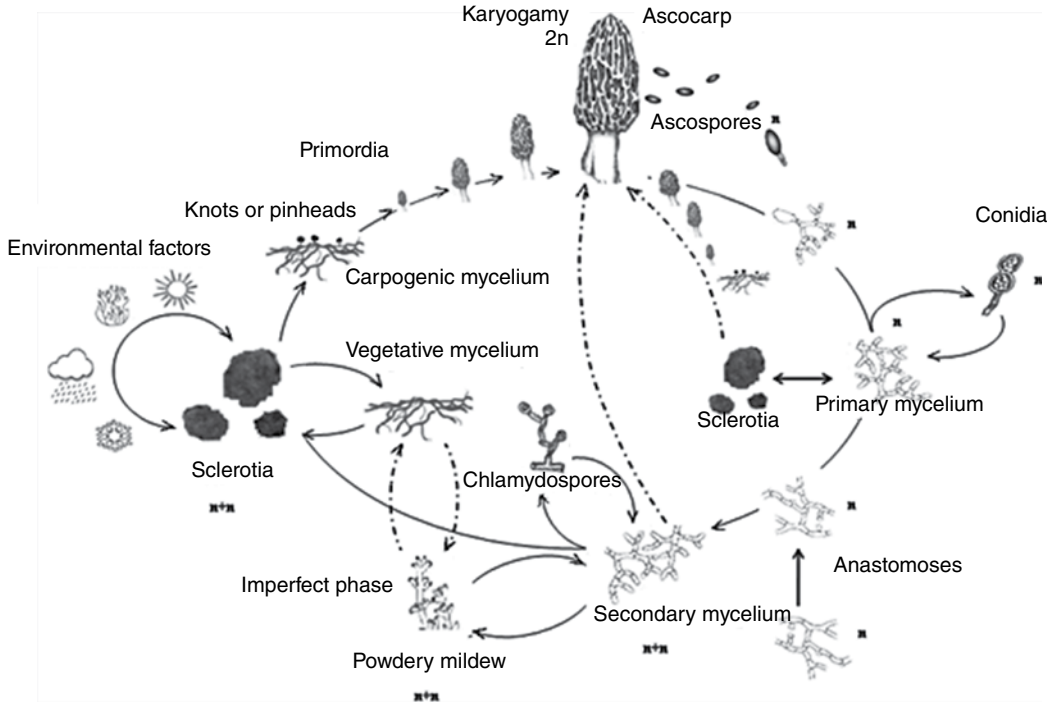

Figure 3.3 Theoretical life cycle of the genus *Morchella*. Dotted lines indicate possible routes. AlvaradoCastillo, G.; Mata, G.; Sangabriel-Conde, W. Understanding the life cycle of morels (*Morchella* spp.). Revista Mexicana de Micologia, v. 40, p. 47-50, 2014. With pennission.

3.17 Final Considerations

Mycorrhizal mushrooms are an important source of income in the areas where they occur naturally. Due to their importance, these mushrooms have been considered as an important alternative to the exploitation of forest ecosystems by logging. Public policy has allowed forest parks to be harvested in this manner, bringing benefits to the people living around these areas. Unfortunately, the mycorrhizal mushrooms species that are most prized are native to the Northern hemisphere, with its temperate-to-subtropical climate.

Nevertheless, the Southern hemisphere also features countries with climates similar to the mycorrhizal mushroom-producing regions of its Northern counterpart, including Australia, New Zealand, South Africa, Chile, Argentina, and Uruguay (Hall et al., 1998, Deschamps, 2002). In South America, Chile stands out for its wild mushroom export tradition. Chile exports *Suillus luteus* and *S. granulatus*, referred to as *Boletus luteus*, *B. edulis*, or simply *Boletus* (Deschamps, 2002). Brazil is an importer of these mushrooms from Chile, which arrive at supermarkets under the names of "Funghi Sechi" or *Boletus*. The species *S. luteus* was first classified as *B. luteus*, and only later discovered to be of another genus, leading to its reclassification as *S. luteus*. While this could explain ascribing the name *Boletus* to the exported product, certainly there also is great commercial interest in joining these mushrooms with the real *Boletus*. Morels are also produced in Chile, with *Morchella intermedia* being the most known species. The species *Phlebopus bruchii* bears a strong resemblance to the *Boletus*, which led to its initial classification as *Boletus bruchii*, and this similarity allows it to achieve twice the price

of the *Suillus* on the South American market. Important species such as *Lactarius deliciosus* and *Ramaria* spp. are also now common to Chile, as well.

In addition to Chile, Argentina and Uruguay also contain geographical regions with the potential to support the production of these mushrooms. In Uruguay, species such as *Tricholoma sulphureum*, *Lactarius deliciosus*, and *Suillus granulatus* occur naturally, while in Argentina, these last two are found in addition to *Phlebopus bruchii*, a type of mushroom highly appreciated in the Argentinian capital. As a result, Deschamps suggested that the countries of the Southern cone should invest in the exploration of *Phlebopus bruchii* species associated with the tree species *Fagara* coconut; and *Lactarius deliciosus*, *Suillus granulatus,* and *S. luteus* associated with species of *Pinus*. These species may be more amenable to cultivation in comparison to the mycorrhizal species native to the Northern hemisphere, where until recently, only a few species of *Tuber* have ever been successfully grown in orchards.

While Brazil is considered a tropical country, it exhibits comparative climatic diversity; Southern Brazil has a humid subtropical-to-temperate climate. In colder areas, the temperature drops below zero during the winter, and snow precipitation occurs in mountainous areas. As such, this region of Brazil would be an excellent location to attempt the introduction of natural production of mycorrhizal mushrooms. Sobestiansky (2005) carried out a survey of naturally occurring species in the region and reported, among many others, *Lactarius deliciosus*, *Suillus luteus*, *Boletus edulis*, and *B. brasiliensis*. In addition, several Brazilian institutions have a long tradition of studying mycorrhizal fungi associated with *Pinus* and *Eucalyptus*. Among the fungi associated with *Pinus*, the *Suillus* genus has been found to have a high occurrence of fruiting bodies in pine reforestation areas. While these areas in Brazil could be lucrative harvesting regions, the lack of wild mushroom harvest traditions among the Brazilian people presents a barrier to immediate adoption, as Brazilian people may be afraid to collect poisonous mushrooms by mistake. Investment from government agencies in cooperation with tourism agencies for the training of professionals in the identification of these species for harvesting purposes could help promote a major shift in this direction, as Brazilian consumers have shown an increasing interest in the consumption of mushrooms. In this context, wild mushrooms could have great cultural and tourist appeal, bringing additional economic benefits to mushroom-producing regions.

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4

Genetic Aspects and Strategies for Obtaining Hybrids

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The history of improvement of any species is linked to its domestication, cultivation, and commercialization. Initial gains can be made by developing good cultivation techniques and selecting strains from the existing variability. For enhancing productivity as well as quality and develop quality planting material it becomes necessary to combine characters from different sources. Mushrooms are fruit bodies of fungi and in the last century many edible species of mushrooms have been brought under cultivation. World production at the beginning of the twentieth century was very low and restricted to very few species of which the button mushroom was the major contributor. In the last few decades, a number of other species have gained popularity and the share of the button mushroom has been in decline. In the last four decades, there has been exponential growth in production of button (Figure 4.1) and also other mushrooms (Figure 4.2). In 2010 the species-wise share of six mushrooms to total world mushroom production was 30% *Agaricus* (button), 27% *Pleurotus* spp (oyster), 17% *Lentinula* (shiitake), 6% *Auricularia* (wood ear), 5% each of *Flammulina* (winter), and *Volvariella* (paddy straw). These six account for 90% of global mushroom production (Royse, 2014). The relative contribution of different species is changing rapidly as presented in chapter 2.

Fungi are a separate kingdom, have unique traits, and are the second largest group. James et al. (2006) (70 authors from 35 institutes in 6 countries) analyzed information from six key genetic regions in almost 200 contemporary species to reconstruct the earliest days of fungi and their various relations, and inferred that the three eukaryotic kingdoms, animals, fungi, and plants, diverged more than 1.5×10^9 years ago, with the plant kingdom diverging slightly earlier from the other two. Plants split from animals about 1.547 billion years ago, fungi split from animals about 1.538 billion years ago. This means fungi split from animals just 9 million years after plants did. Fungi invaded land about 500 million years ago – much before land plants. Fungi are eukaryotes, nearly all multicellular (yeasts are unicellular), distinguished from other kingdoms by nutrition, structural organization, growth, and reproduction. In the past, slime molds (myxomycetes) and water molds (oomycetes) were also classified as fungi, but their cell wall has cellulose and lacks chitin whereas the cell wall of true fungi is made of polysaccharides and chitin. Molecular studies show that all true fungi are from a monophyletic clade, that is, all share a common ancestor. Fungi have been further classified into various phyla that include Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota. Most mushrooms belong to the class Basidiomycetes of the latter phylum. Genetic aspects of these phyla have been identified and much work has been reported on Ascomycetes like

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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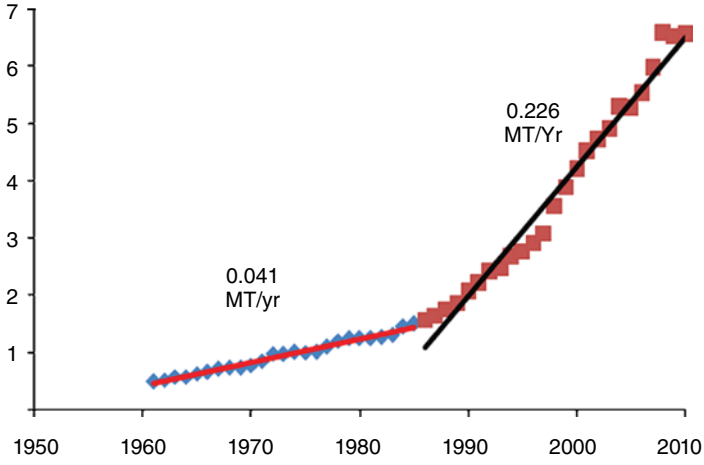

Figure 4.1 Global mushroom production (based on data from FAOSTAT, 2011).

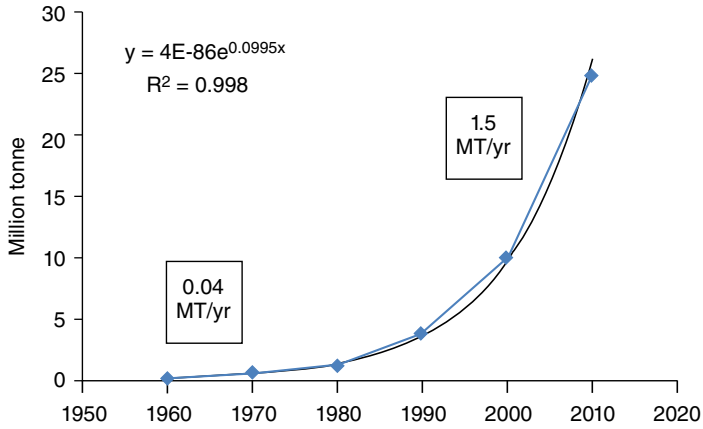

Figure 4.2 Global mushroom production over the last four decades.

Saccharomyces cerevisiae, *Neurospora*, and basidiomycetes like *Schizophyllum commune*. We, however, will focus on the commercially important mushrooms and in the present chapter we will mainly focus on improvement of button and oyster mushrooms.

4.1 *Agaricus bisporus*

4.1.1 Introduction

The first breeders of button mushrooms were the French in the seventeenth century who first cultivated button mushroom and started selecting the desirable types. The selection process has continued since then and a major step has been the selection of white colored mutant. In 1926 a pure white mutant occurred on the beds of Low Downing of Downing town, PA, and Lambert propagated it by means of multispore culture. Following three generations of multispore culturing and selection on color, a pure "white strain" was obtained (Lambert, 1959). Now there are four clearly differentiated groups of strains within the type *Agaricus bisporus*, specifically "white strains," "off-white strains," "hybrid strains" (i.e., hybrid between white and off-white strains), and "brown strains" (Fritsche and Sonnenberg, 1988). White strains can be

further divided into rapid and slow fruiting strains. This simple selection in 1926 has had a vast impact on subsequent cultivation and improvement programs and the name white button mushroom itself reflects the significance of this trait.

There have been three major turning points in the history of cultivation of this species. One was at the beginning of the twentieth century when pure culture was prepared and it became possible to propagate pure spawn. The second change was the introduction of the short method of composting that helped in devolving standard cultivation practices. The third and most important step, the production and use of hybrids, had to wait until the 1980s. Sinden's grain spawn, Hauser's composting technology, and Fritsche's hybrid development have played a pivotal role in the growth of this important mushroom.

Hybridization in button mushrooms was considered impossible or difficult till 1980. Dr James W Sinden in his presentation on "Strain Adaptability" at the Second North American Mushroom Conference, Toronto, Canada, August 1980 stated that,

> As we know, hybridization as recognized in other plants and animals and even some fungi, was either not possible or so difficult as to discourage the would-be breeders. Throughout the years there have been attempts by enthusiastic geneticists of the fungi to attack the problem of new variety development by crossing the known ones, but ever since Dr Lambert showed that our mushroom was perfectly capable of reproducing itself by single spore cultures, the hopes of crossing have been dim. Reports appear in the literature from time to time heralding success in crossing. Several I know to be based on false observations and others I suspect. At least no commercially useful hybrid strain has so far been developed. My own assessment of the situation is that even if hybridization by some special complicated treatment such as is now available for crossing other fungi and plants was successful, the chances of producing a commercially advantageous strain would be very questionable.

The first hybrids were commercialized by Darlington in November 1981 (Fritsche, 1981). In the Sinden Award Lecture of 1985, Dr Gerda Fritsche described the breeding strategy used for the development of Hybrid Horst U1 and U3 line (Fritsche, 1986). It is evident that the initial impact on button mushroom cultivation was due to refinements in cultivation methods and actual hybridization is a relatively new achievement in button mushroom cultivation. Some of the reasons for delay in hybridization in the button mushroom was lack of understanding of reproductive biology and availability of limited germplasm.

4.1.2 Understanding Reproductive Biology

The degrees of selfing or outcrossing rates affect choice of breeding strategy and method of germplasm collection in higher plants. Sexual reproduction is an integral step for survival in nature through generation of variability and getting rid of genetic load. The three cardinal events in sexuality are; (1) plasmogamy, that is, fusion of two haploid cells causing two nuclei to be brought together into the same cell, (2) karyogamy, that is, fusion of two haploid nuclei, and (3) meiosis, the production of haploid nuclei. Meiosis is a two-stage division during which variability is generated in both meiosis I and meiosis II. The first stage is a reduction division where chromosome number in the two nuclei is reduced to half and variability is generated due to random distribution of chromosomes from the parents. The second division is like mitotic division in which chromatids separate and variability generated through crossing over of chromatids at chromosome pairing stage is released. As a result, each of the four nuclei produced after meiosis contains a different combination of alleles (Chapter 3 discusses specific issues in mushroom biology).

Normally in Basidiomycetes, the class to which most mushrooms belong, basidia produce four spores, each having one haploid nucleus. The mycelium produced by each uninucleate spore has cells all of which are of same mating type. Such mycelium is referred as monokaryotic. When two compatible monokaryotic mycelia fuse, the result is a mycelium in which each cell has two nuclei of the opposite mating type. This is referred to as dikaryotic mycelium and the dikaryotic stage in Basidiomycetes is maintained by formation of clamp connections that help in synchronized mitosis. The presence of clamp connections is a unique feature of Basidiomycetes. *Agaricus bisporus*, however, differs from this normal cycle in many ways. Here instead of four spores, basidia produce only two spores. Various studies have shown that nuclear migration is not random and each spore has two nuclei of the opposite mating type. As a result, the spore can germinate, survive, and multiply further by producing fruit bodies. A few of the basidia are trisporic or tetrasporic that produce spores carrying only one nucleus. On germination, the spores having only one nucleus produce mycelium whose cells are multinucleate, i.e., have many nuclei. Such mycelium carrying nuclei of the same type is referred as homokaryotic mycelium. When two homokaryotic mycelia carrying compatible mating factors fuse, the result is heterokaryotic mycelium. Here too, the cells are multinucleate and the clamp connections, the unique feature of a basidiomycete, are absent (Figure 4.3). Hence there is no way to morphologically differentiate homo- and heterokaryotic mycelia. The simple though

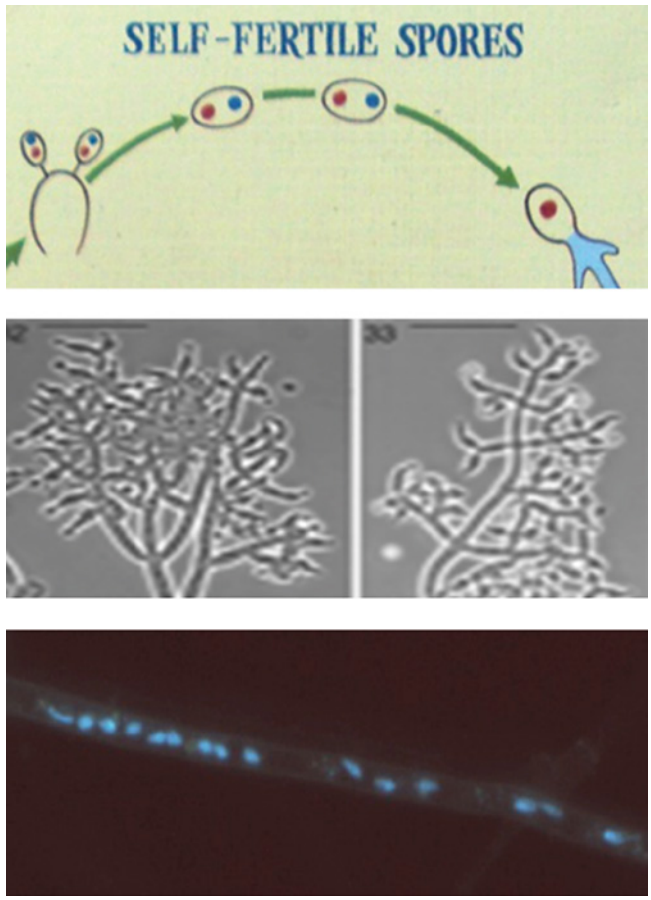
SELF-FERTILE SPORES
Secondary homothallism, only a few spores are non-fertile
Absence of clamp connections, no way to differentiate homo- and heterokaryotic mycelia
Both homo- and heterokaryotic mycelia are multinucleate

Figure 4.3 Three factors impeding hybridization in button mushrooms.

time-consuming method is to test each culture for fruiting as only heterokaryotic ones will produce fruits. Mycelium that does not produce any fruiting body is referred as non-fertile and it generally indicates that the culture is homokaryotic. However, this may not always be true as there are a number of genes that govern differentiation of vegetative mycelium into fruiting body and mutation in any of these can cause non-fruiting behavior. A number of other methods and approaches are now available. For example, we can look for markers. Heterokaryons are likely to have various polymorphic loci whereas homokaryons will have only one allele. Such polymorphism by considering number of loci can help in differentiating the homo- from heterokaryotic mycelia. These aspects are discussed later in the chapter.

Sex determination in fungi is controlled by a small, specialized region of the genome in contrast to the large sex-specific chromosomes of animals and some plants. Different gene combinations reside at these mating-type (MAT) loci and confer sexual identity. In most fungi, sexual reproduction is bipolar; that is, two alternate sets of genes at a single MAT locus determine two mating types. However, in the Basidiomycota, a unique (tetrapolar) reproductive system emerged in which sexual identity is governed by two unlinked MAT loci, each of which controls independent mechanisms of self/nonself recognition. Bifactoriality has therefore most likely evolved in Basidiomycota after their divergence from Ascomycota. Tetrapolar-to-bipolar transitions have occurred on multiple occasions in the Basidiomycota, resulting, for example, from linkage of the two MAT loci into a single inheritable unit. Evolution of uni- and bifactorial sexual compatibility systems in fungi has been reviewed by Nieuwenhuis et al. (2013). Bifactoriality reduces the odds of compatibility (25% instead of 50% in unifactorial species) among the haploid products of a diploid individual and could therefore promote outcrossing/reduce selfing (Hsueh et al., 2008; Billiard et al., 2012). A homothallic fungus produces gametes that are compatible with all other individuals, including its own mitotic descendants, most often by expressing both mating types (Lin and Heitman, 2007). This leads to the possibility of same-clone mating, which does not allow recombination between different genotypes while still incurring the costs of sex. Homothallism thus comes with potential costs (Giraud et al., 2008; Billiard et al., 2011; Billiard et al., 2012). Pseudohomothallism has been intensively studied in *Neurospora tetrasperma*, where it was first described (Dodge, 1927). This might be considered as a form of reproductive "assurance" allowing unhindered sexual reproduction even in the absence of a suitable mating partner (Merino et al., 1996).

4.1.2.1 Different Mating Systems in the Genus *Agaricus*

The *Agaricus* species have bipolar (or unifactorial) breeding systems (Elliott, 1978, 1979a) and within the genus, different species exhibit a range of diverse life-styles (Kerrigan et al., 1993), homothallism (e.g., *Agaricus subfloccosus*), pseudohomothallism (e.g., *Agaricus bisporus*), and heterothallism (e.g., *Agaricus bitorquis*). Earlier efforts to characterize mating-type genes in *Agaricus* species (Li et al., 2004) were severely constrained by the lack of genome sequence. The *A. bisporus* genome sequence has enabled comprehensive mapping of the genes encoding homeodomain transcription factors in this pseudohomothallic species, and revealed genes encoding conserved pheromone and pheromone receptors (Morin et al., 2012).

4.1.2.2 Different Mating Systems within *Agaricus bisporus*

Agaricus bisporus (Lange) Imbach, the button mushroom, has a unifactorial mating system (Miller and Kananen, 1972) with multiple alleles (Imbernon et al., 1995) at the MAT locus on chromosome I (Xu et al., 1993). Three varieties have been reported in *A. bisporus* viz., *Agaricus bisporus* var. *bisporus, A. bisporus* var. *burnettii*, and *A. bisporus* var. *eurotetrasporus*. These varieties differ in their life cycle, spore size, and average spore number per basidium

(Callac et al., 2003). In fact, the three sexual reproductive modes known in the Basidiomycetes (heteromixis, intramixis, and homomixis) exist in *A. bisporus*.

Agaricus bisporus var. *bisporus* has a multiallelic unifactorial system of sexual intercompatibility (Miller and Kananen, 1972), the locus MAT (Xu et al., 1993) having 14 alleles (Kerrigan et al., 1994; Imbernon et al., 1995), and its life cycle is amphithallic, that is, pseudohomothallic (= secondary homothallic) or heterothallic (Figure 4.4). In this variety, most of the basidia are bisporic and produce heterokaryotic spores that confer upon it a predominant pseudohomothallic life cycle (Raper et al., 1972). More precisely, for 215 wild French isolates examined in cultivation, Callac et al. (1996) found that the percentages of bi-, tri- and tetrasporic basidia were on average 81, 18, and 1%. Theoretically it means that more than 18% of the spores can be homokaryotic. The ploidy status of the spores of the three-spored basidia is unknown, and the

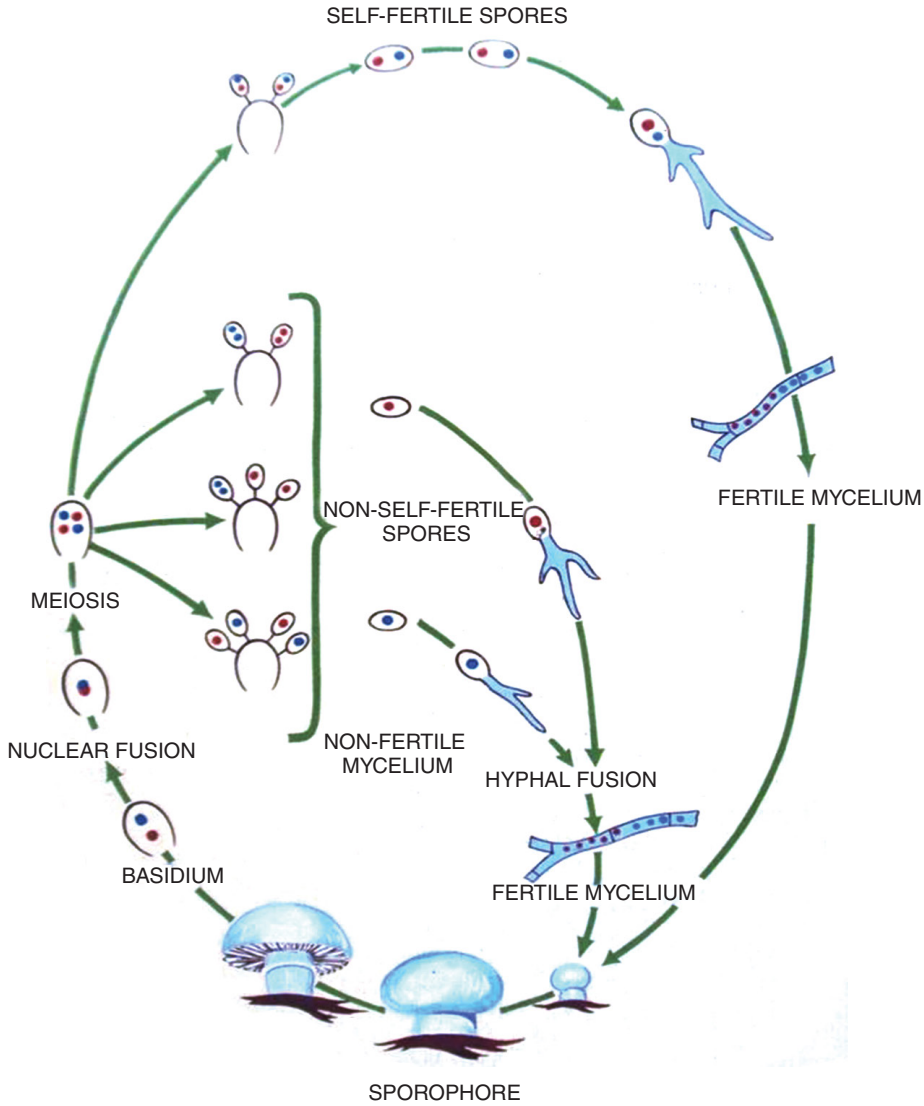

Figure 4.4 Life cycle of *Agaricus bisporus* var. *bisporus*.

Table 4.1 Variation in type of basidia in two varieties of *Agaricus bisporus* (button mushroom).

Variety	Collections from	No. Analyzed	Percentage of Basidia that Were…		
			Bisporic	Trisporic	Tetrasporic
bisporus	France	215	81	18	1
burnettii	California	58	1	14	85

Based on Callac et al. *(*1996*)*

germination rate of the homokaryotic spores and/or their viability can be lower than those of the heterokaryotic spores and hence the actual proportion of homokaryotic mycelia can be less (see review by Savoie et al., 2013). The low percentage of homokaryotic offspring is a significant drawback, slowing down the breeding work (Kerrigan et al., 1992). Most of the wild populations and all the traditional cultivated strains belong to *A. bisporus* var. *bisporus*. In this variety, most basidiospores receive two non-sister postmeiotic nuclei carrying different mating-type alleles that give rise to heterokaryotic fertile mycelia and tend to maintain the parental genotype in the offspring (Pelham, 1967; Summerbell et al., 1989; Kerrigan et al., 1993). Thus, the secondary homothallism with migration of non-sister nuclei into a spore and with no or scanty crossing over will result in fixation of heterozygosity, which in self pollinated higher plants like wheat has been achieved by polyploidy.

A. bisporus var. *burnettii* Kerrigan and Callac has been described on the basis of specimens found in the Sonoran Desert of California (Callac et al., 1993). In the sporophores of this variety, most of the basidia are tetrasporic which means that its amphithallic life cycle is predominantly heterothallic (Kerrigan et al., 1994). More precisely, for 58 wild Californian isolates examined in cultivation, Callac et al. (1996) found that the percentages of bi-, tri-, and tetrasporic basidia were on average 1, 14, and 85 (Table 4.1). This variety differs from the two other varieties by traits reflecting adaptation to dryness, smaller mean spore size, and faster sporophore development. The average spore numbers per basidium are primarily determined by the BSN locus (basidial spore number), which is linked to MAT on chromosome I (Imbernon et al., 1995, 1996; Callac et al., 1997).

A. bisporus var. *eurotetrasporus* Callac and Guinberteau was described on the basis of rare tetrasporic specimens found in France and in Greece (Callac et al., 2003). The life cycle of this variety is homothallic: homokaryotic sporophores produce homokaryotic spores, giving rise to fertile homokaryons. The basidia are mainly tetrasporic as in var. *burnettii*, but spores have the same mean size as those of var. *bisporus*. Callac et al. (2003) reported one strain of this species from Greece and two strains from France.

Homothallic species may originate from heterothallic predecessors, and the homothallic life style may have a selective advantage under certain ecological pressures. This hypothesis is consistent with the repeated occurrence of homothallism within numerous genera and the fact that many heterothallic fungi achieve homothallism in the form of pseudohomothallism by mating-type switching or packaging two compatible nuclei into one spore. Homothallic individuals contain all the necessary genetic information for full sexual expression (Lin and Heitman, 2007).

4.1.3 Understanding and Collecting Variability

4.1.3.1 Distribution

Agaricus bisporus is a secondary decomposer fungus and grows well on partially decomposed litter on forest floors and grassland soils rich in humic substrates. It is found growing over a

wide geographical range on leaf and needle litter in temperate forests of *Picea* (Spruce) (Alberta, Canada; Washington, USA), *Cupressus* (Cypress) (California, USA, Greece, Crete, Italy, France), *Juniperus* (Mexico), *Prosopis* (Mesquites), *Tamarisk* (Sonoran Desert, California), mixed Montane forest (New Mexico), *Eucalyptus* (Israel, Morocco, Congo), and in other settings including pastoral land use areas (UK, Russia, China, Australia, Tasmania, Argentina) (Kerrigan et al., 1995; Xu et al., 1997). Geographically it has been mainly reported from Europe, the Mediterranean region, and North America. Some wild specimens have also been isolated in other areas such as Asia. For instance, wild *A. bisporus* strains were collected from the Tibetan Plateau (Wang et al., 2008a). The known geographic range of *A. bisporus* extends from the boreal region of Alaska (Geml et al., 2008) to the equatorial climate of Congo (Heinemann, 1956), and from coastal dunes to mountains of more than 3000 m elevation (Largeteau et al., 2011a) (see Savoie et al., 2013).

4.1.3.2 Germplasm Collection

A prerequisite for breeding is the availability of genetically diverse germplasm. In the 1980s, there appear to have been fewer than 20 independent lines of *A. bisporus* in mainstream culture collections worldwide, including those of commercial laboratories (Kerrigan, 1996). There was concern about losing genetic diversity forever, and at the end of the 1980s, a few researchers decided to support collection and conservation of the germplasm of *A. bisporus*. Two major collections, the ARP (Agaricus Resource Program; Kerrigan, 1996) in the USA, and the CGAB (Collection du Germoplasme des Agarics à Bordeaux; Callac et al., 2002) at INRA-Bordeaux represent more than 800 wild specimens, mostly collected either on cypress or spruce litter, or on manure, but also in sandy semi-arid habitats. Their distribution mainly covers Europe, the Mediterranean region, and North America. In China, Wang et al. began to gather *A. bisporus* germplasm resources from all over the world since 1983 (Wang et al., 2008), and have collected more than 400 strains of *A. bisporus* including 206 cultivars, 168 foreign wild strains, and 61 Chinese wild strains. In India, we started collecting germplasm from various sources including ARP and now have 137 accessions at the Directorate of Mushroom Research, Solan.

Germplasm collection has to be a continuous process and with changes taking place across the world like deforestation, urbanization, increasing human population, indiscriminate collection of mushrooms from the wild in some areas, and climate change, there is going to be an impact on natural diversity and hence the need for collaborative programs across the world. Despite the market share of the button mushroom the emphasis on germplasm collection is inadequate.

4.1.3.3 Natural Variability

There is spatial and temporal genetic heterogeneity in natural populations of *A. bisporus*. In a study at two sites in France, one disturbed regularly and the other free of human disturbance, it was seen that both sites contained high levels of genetic diversity; there was little evidence for extensive vegetative clonality; and contrary to expectations, there was very limited evidence of pseudohomothallic reproduction. Results from tests of Hardy–Weinberg equilibrium and genotypic equilibrium showed that outcrossing and recombination have played significant roles in both populations (Xu et al., 2002). It implies that in nature variability in fungi is not necessarily a function of mode of sexual reproduction.

Xu et al. (1997) undertook genetic analysis of a collection of 342 isolates from 12 locations (214 from North America and 128 from Eurasia + 16 cultivated strains) for 10 nuclear loci marked by restriction fragment length polymorphisms (RFLPs), and found high genetic diversity in all geographic populations. Based on mitochondrial DNA the researchers divided these isolates into two subsets; one set of 108 "cultivar-like" (that carry either of the two mitochondrial

DNA haplotypes found in cultivated strains) and the other set having 234 isolates (that carry a diversity of mtDNA haplotypes not found in cultivated strains) and found little variability in the former and high variability in the latter. This high diversity in natural populations suggests that there is a wealth of genetic material in nature available for both genetic studies and mushroom breeding. Some economically important traits (e.g., bacterial blotch resistance, resistance to degeneration, and tetraspory) have been identified in this collection of isolates (Callac et al., 1993; Li et al., 1994).

Studies on wild germplasm have produced data on phenotypic diversity for morphological traits and behavioral traits. Trait diversity in wild *A. bisporus* was reviewed by Kerrigan (2004). There have been a number of reports where variability in wild accessions has been much higher than in the cultivated strains. Microsatellite markers and SNPs have been used to demonstrate the close relationship between the cultivated strains of *A. bisporus* and also evaluate the available genetic diversity in collections. In 19 wild accessions on average for 29% of all 600 SNP markers, both alleles of the cultivar strain used as reference were found by Sonnenberg et al. (2011) while the percentage was about 50% for non-hybrid cultivars. Using 33 SSR markers, Foulongne-Oriol et al. (2009) observed a significantly higher polymorphism among 20 wild isolates than among seven cultivars representing the six morphotype lineages assumed to represent all or almost all the genetic variability available among the traditional cultivars and the hybrid Horst U1. DNA analysis of commercial cultivars of *A. bisporus* in India revealed genomic homogeneity and single nucleotide polymorphism in 5.8s ribosomal RNA gene spacer region (Yadav et al., 2007).

4.1.4 Utilizing Variability

Variability collected from nature can be analyzed and used directly. In fact, most of the early introductions are the result of such an approach. There can be direct introductions after selecting suitable strains from the existing variability, or there can be efforts to purify. Rejuvenate or improve the planting material by repeated sub culturing, tissue culture, multispore culture, and so on.

4.1.4.1 Direct Selection of Natural Variants

One of the earliest examples of direct selection is the selection of white mutants in 1926 as described earlier. In India, from the early 1960s various exotic strains of *A. bisporus* were introduced after evaluation under different geographical locations, various substrate formulations, growing conditions, and so on (Seth, 1973; Kaul and Kachroo, 1975; Kumar, 1976; Kumar and Chandra, 1983). Cultivation in the early days was done on long method compost, and mushrooms were being grown under natural conditions. Some of the early strains that were selected for commercial cultivation in India were strain S-11 in Himachal Pradesh and strain RRL-89 in Kashmir (Seth, 1973; Kumar, 1976; Kaul and Kachroo, 1975). In India, breeding work was initiated in the late 1980s (Bhandal and Mehta, 1989) and the first hybrid of the button mushroom was released in 1997. The scenario world over was the same and most of the strains in use before 1980 were direct introductions maintained using tissue or multispore culture.

4.1.4.2 Tissue Culture, Multispore, and Single Spore Cultures

Tissue culture: In fungi propagation of culture from a tissue or a spore from a fruiting body is the method of clonal multiplication. Tissue culture as a technique for developing pure culture is important while preparing culture from wild collections. Fast growing tissue cultures of mushrooms are maintained by periodic transfer of mycelium on suitable medium for future use. In *A. bisporus*, tissue culture technique for improvement or culture maintenance was not found

reliable as growth was slow in some cultures made using tissue culture and there were undesirable variations and lower yield than parental strains in many of such cultures (Sarazin, 1952; Fritsche, 1966; Bhandal and Mehta, 1989). Mehta (1991) recorded that pileus cultures showed the minimum variability and was more productive in comparison to cultures obtained from stipe and gill.

Multispore cultures: Multispore cultures have been used to maintain cultures and even improve them. In 1928, Lambert selected the white variety after three generations by this method (Lambert, 1959). Germination of spore mass results in a uniform and stable mycelium. It was also reported that multispore cultures showed better, worse, and the same yield as the parental spawn. A multispore selection A-6 was selected in 1932 and it was recovered as an improved strain in 1960 after testing of 140 spore prints over the years (Fritsche, 1981; Sinden, 1981). There are various reports suggesting that multispore cultures of *A. bisporus* were variable in yield and showed faster degeneration in comparison to parental cultures.

Single spore isolates: Variation in single spore isolates was observed by number of researchers and a number of varieties in the market are the outcome of this simple approach. The approach is still in use because of its simplicity. Our earlier studies (Bhandal and Mehta, 1989) and many more before that showed a high degree of variability. Such cultures are considered more stable than the multispore cultures. Recently, the Directorate of Mushroom Research, Solan, has released two high yielding single spore selections (Figure 4.5) (DMR, 2015; Singh et al., 2016).

Based on molecular studies there have been viewpoints to treat such isolates as essentially derived varieties. Many researchers believe that in single spores, due to migration of non-sister nuclei and hardly any crossing over, heterokaryons produced from single spores of a single strain carry the same genetic constitution, though genetic material may be distributed a bit differently due to random migration of chromosomes during meiosis I. The extent of variation observed in single spore isolates suggests that other mechanisms are at play. Furthermore, as a breeder our interest is in variation in few economic traits whereas molecular techniques reflect variation in DNA, the major part of which is non-functional. From a breeding point of view this approach will continue to be used particularly after developing hybrids. In fact, it may be desirable to develop new combinations after hybridization. A close scrutiny of the literature indicates that U3 was also a single spore isolate of the hybrid U1 (Figure 4.6).

Figure 4.5 Crop of DMR-03, a single spore isolate, at a commercial unit. (*See color plate section for the color representation of this figure.*)

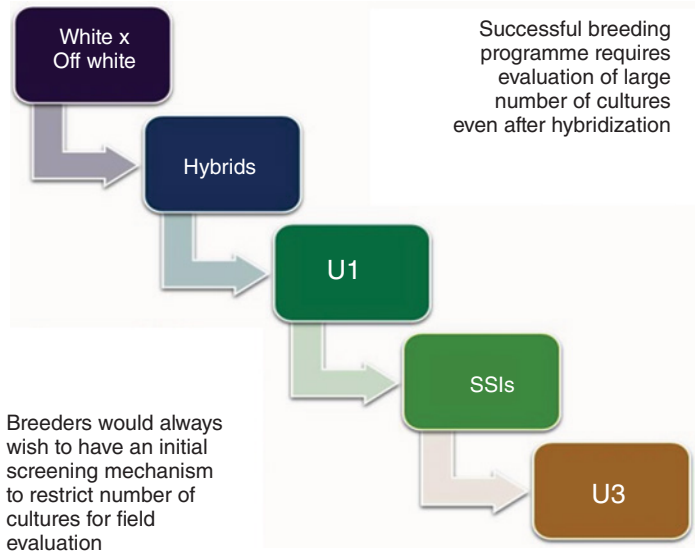
White x
Off white
Hybrids
U1
SSIs
U3
Successful breeding programme requires evaluation of large number of cultures even after hybridization
Breeders would always wish to have an initial screening mechanism to restrict number of cultures for field evaluation

Figure 4.6 Steps in development of U1 and U3.

4.1.4.3 Mutations by Ionizing Radiation or Chemical Mutagens

In the absence of sufficient germplasm basidiospores, non-sexual spores or hyphal segments can be exposed to physical or chemical mutagens to create variants. The application of this approach for isolation of auxotrophs is more useful and such variants can help in understanding genetics of various traits. Hui (1989) exposed protoplasts of *Pleurotus florida* and *Flammulina velutipes* to gamma rays and demonstrated that this approach of using protoplasts of edible fungi for radiation breeding is a more rapid, simple, and effective method as compared with conventional methods of irradiating spores. This approach has found little applicability to button mushrooms.

4.1.4.4 Strain Mixing to Achieve Hybridization

To generate new combinations, it is necessary to combine traits through intermating two different strains. The first thought that may come to mind to hybridize strains is to mix the two strains. Kneebone et al. (1972) attempted to obtain new strains by growing strains together on grains and shaking them at regular intervals. Stoller (1974) attempted to induce fusion of mycelial colonies by adding chemicals (nicotinic acid) in submerged cultures and stirring with a magnetic stirrer. Fritsche (1983) also attempted spawning with mixtures of two strains. Even when we started our breeding program in the 1980s, this was one of the first approaches we attempted (Bhandal and Mehta, 1989). Sinden (1981) also explained his efforts to obtain hybrids by mixing strains. It may be possible to get a hybrid but directed and repeatable hybridization may not be possible. In almost all cases the success was limited and there was hardly any utility of such an approach for achieving targeted results. Systematic hybridization required proper understanding of mating systems and genetic parameters.

4.1.5 Exploiting Variability Through Hybridization

Cross breeding is one of the most effective methods of combining desirable characters of various strains into a new one. The major problem in cross breeding in this species is its secondary homothallic nature. Thus, various approaches such as use of non-fertile spore cultures, nutritional auxotrophs, drug resistance, enzymatic markers, molecular markers, protoplast

fusion, and so on, have been suggested by various researchers. Initial gains may be possible through direct introductions, tissue culture, multispore or single spore cultures but the gains are limited to the single gene pool of the accession being used. Significant gains are possible only through hybridization where it is possible to integrate characters from two parents. The actual hybridization of button mushrooms started only after clear understanding of the life cycle of this mushroom in the 1970s. The work on crossing white strains and off-white strains started in 1976 at the Mushroom Experimental Station in Horst, The Netherlands that resulted in the development of hybrid strain U1. Another strain U3 was developed from segregates of this hybrid. Few new hybrids with a different genetic background have been developed since then. As a result, all currently grown cultivars are assumed to be related to a limited number of traditional genotypes, and *A. bisporus* appears to be nearly a monolineage crop. The breeding program in India started in 1984 (Bhandal and Mehta, 1989) and we released the first hybrids NCH 102 in 1997 (NRCM, 1997). In continuation of this work, in 2014 we released two browning resistant hybrids of button mushrooms, NBS-1 and NBS-5, by traditional breeding. This involved screening of more than 1000 single spore isolates, identification of 39 self sterile single spore isolates, and their intermating to produce 361 hybrids (Figures 4.7 and 4.8) (DMR, 2015).

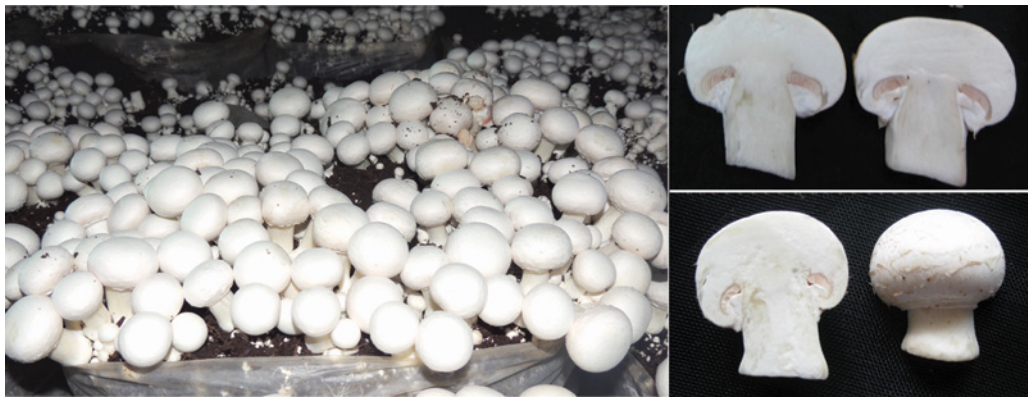

Figure 4.7 Crop of NBS-1 (left), cross section of fruiting body (top right), and quality of fruiting bodies after storage at room temperature for 48 h (bottom right). (*See color plate section for the color representation of this figure.*)

Figure 4.8 Crop of NBS-5 (left), cross section of fruiting body (top right), and quality of fruiting bodies after storage at room temperature for 48 h (bottom right). (*See color plate section for the color representation of this figure.*)

Molecular techniques have helped in overcoming some of the problems of identification of homokaryons, hybrids, or selection of traits of interest in the lab. There have been efforts to develop hybrids in other locations as well; for example, in the 1980s Peng from Taiwan developed the hybrid strain by hybridization between an off-white and snow-white strain for which he had mated 41 slow growing mycelia of off-white strain with 47 of snow-white and obtained 323 successful crosses. The first step in a breeding program is to identify the parental strains whose traits are to be combined. Gains can be greater by choosing diverse parents and having information about inheritance of the traits under question. Diversity has been studied at morphological level using molecular methods and today we have the complete linkage map. A brief discussion of these follows.

4.1.5.1 Genetic Parameters: Heritability, Combining Ability, and So On

For an effective breeding scheme, it is important to understand various genetic parameters. There are only limited reports where efforts have been made to study genetic parameters quantitatively. In fact, there are very few breeding efforts even after complete understanding of the life cycle. Bhandal and Mehta (1989) estimated heritability in the broad sense of yield and related traits based on the data obtained from evaluation trials of strains and single spore isolates. The correlations among traits were also partitioned into direct and indirect effects for better understanding of selection criteria during breeding programs (Singh and Kamal, 2011a). The first study on estimating combining ability using diallel was made by Gao et al. (2013). These researchers studied the genetic variation in bruising sensitivity of *A. bisporus* by intercrossing 19 homokaryotic lines in a diallel scheme. Bruising sensitivity was shown to be a trait with a very high heritability. Estimates of the general combining ability (GCA) for each homokaryotic parental line and estimates of the specific combining ability (SCA) of each hybrid worked out in this case have a significance in breeding programs for higher gains and understanding possible hetrotic effects. Most of the breeding work undertaken in button mushrooms is based on the presumptions without assessing heritability or combining ability.

4.1.5.2 Genome Analysis

A number of researchers have contributed to our knowledge of the *Agaricus* genome Starting with Evans' work in 1959, when the haploid chromosome number was established at 13 using light microscopy, the genome was sequenced in 2012 by Morin et al. with contributions by a number of authors in between. The first fungal genome that was sequenced in 1996 was of the yeast *Saccharomyces cerevisiae*. At 12.2 Mbp, it is about one-third the estimated size of the *A. bisporus* genome. Approximately 6000 genes have been identified on 16 chromosomes. The first genome sequence of a filamentous fungus, that of *Neurospora crassa*, was published in 2003. This genome, at 43 Mbp, is larger than that of *A. bisporus*, and is believed to incorporate about 10,000 genes (Kerrigan, 2011). Morin et al. (2012) presented two *A. bisporus* genomes, their gene repertoires, and transcript profiles on compost and during mushroom formation. In 2007 the Joint Genome Institute, from the US Department of Energy (DOE), agreed to sequence the whole genome of *A. bisporus* (Kerrigan, 2011). The two homokaryons (haploid genome) of *A. bisporus* studied were H97, obtained from the historically cultivated stock Horst U1 (a bisporic variety belonging to *A. bisporus* var. *bisporus*), and JB137-s8, belonging to the tetrasporic var. *burnettii*. Both genomes are now in the public domain on the JGI's portal web site (http://genome.jgi-psf.org). The genomes of both homokaryons of the first hybrid Horst U1 have now been sequenced. As already mentioned, one of the parental homokaryons of Horst U1, that is, H97, was sequenced by the Joint Genome Institute (http://genome.jgi-psf.org). The other parental homokaryon, that is, H39, has been sequenced by ServiceXs (www.servicexs.com/) using the next sequencing generation techniques of Illumina (Sonnenberg et al., 2011).

The size of the genome and scaffolds generated correlate with the previous observation on genome size (31 Mb) and number of chromosomes (13) in *A. bisporus* variety Horst U1 (Sonnenberg et al., 1996).

The complete genome sequence is an important resource. It has various applications, one of which developed by us is identification of WRKY factors (Kamal et al., 2014). With changing environmental conditions, organisms can reprogram their transcriptome through transcription factors. WRKY transcription factors are a class of sequence-specific DNA binding transcription factors found almost exclusively in plants and are key regulators of gene expressions. The *Agaricus* genome showed the presence of WRKY domain at multiple sites and also different types of WRKY domains could be identified in the genome. Additionally, the NBS (associated with WRKY domain) primer for disease resistance gene amplified fragment in the *A. bisporus* showed the presence of disease resistance genes in the genome. This is the first report of the presence of WRKY domain (specific to plants) in the *A. bisporus* genome. WRKY factors have been studied in detail in plants where these can play an important role in disease resistance, gene silencing, and combating various biotic and abiotic stresses (Pandey and Somssich, 2009).

4.1.5.3 Variation in Isoenzymes, RAPD, RFLP, and Other Molecular Traits

Since the early 1970s, genetic markers such as allozymes, auxotrophic lesions, and morphologic aberrations have been reported in *A. bisporus*, enabling an initial genetic characterization of the cultivated mushroom (Raper et al., 1972; Royse and May, 1982; Spear et al., 1983). With the development of molecular biological tools such as RFLPs and randomly amplified polydisperse DNA sequences (RAPDs), DNA markers for *A. bisporus* became available, which allowed a more in-depth study of the genetics (Castle et al., 1987; Williams et al., 1990). These DNA markers have been utilized as indicators of genetic diversity (similarity) by generating "DNA fingerprints" of individual mushroom strains (Loftus et al., 1988; Horgen et al., 1991; Khush et al., 1992) and have clearly indicated that current strains contain limited genetic variation. Variations in germplasm available to us have previously been studied in detail (Yadav et al., 2003). We produced 361 hybrids using non-fertile isolates from 11 strains of which five high browning resistance strains were finally selected. These five hybrids were characterized using inter-single sequence repeats (ISSR), SSRs, and retro-element based markers. For REMAP studies, we have used successful IRAP primers along with microsatellite primers. These retro-element based markers have shown very high resolution between the parents and hybrids. We have also got a number of fertility linked markers in *Agaricus*, strain specific markers in different strains of *Agaricus*, and also genus and species specific markers in different mushrooms. Retro-element based markers showed high diversity in *A. bisporus*, where diversity is always revealed to be very low with most of the other marker systems (Kamal et al., 2016).

4.1.6 Identification of Parental Strain

The first step in hybridization is to identify the right strains whose characters we wish to combine. The initial selection may be based on visual parameters, but it is important to understand the genetic diversity in the two selected strains. Further, to go for marker aided selection, the knowledge of genome and location of genes/quantitative trait loci (QTL) is also important. Fortunately, now we have much better knowledge of genome of *Agaricus* as compared to the 1970s when breeding programs were initiated.

4.1.6.1 Economically Important Traits

When identifying a parent, we have to look into the genetic background of the parental strains and also the characters proposed to be combined. Initially we can study these traits at

phenotypic level. Now it is possible to map these. Many of the economic traits are quantitative and QTL have been identified. Thus, using molecular techniques, it may be possible to screen parental strains and the homokaryons (= parents of the hybrid) and select for various traits before evaluation. Studies on wild germplasm have produced data on phenotypic diversity for morphological traits and behavioral traits. The main characteristics that have relevance to economic development of *A. bisporus* cultivation are cap color, post-harvest quality reaching consumer expectation, temperature tolerance, disease resistance, and differences in cultivation characteristics such as time of fruiting or number and weight of fruiting bodies (Kerrigan, 2004). There have been a number of studies where diversity at molecular level using RAPD, RFLP, and other molecular markers has been demonstrated. This variability can help to identify parental strains from diverse backgrounds, even though it may not be a reflection of differences in the traits of interest. Some of the important traits are presented in the following subsections.

4.1.6.1.1 Yield and Fruit Morphology

As in other crops, yield and yield-related traits like average weight per mushroom, number of fruiting bodies per m^2, earliness, and cap color are important traits to be selected for. Despite the importance of the trait, the selection process in field trials is not easy due to environmental impact and difficulties in evaluating large accessions. The complex genetic architecture of yield-related traits has been disentangled through QTL mapping (Foulongne-Oriol et al., 2012a). These researchers detected a total of 23 QTL for seven yield-related traits. Four regions underlying significant QTL controlling yield, average weight, and number were detected on linkage groups II, III, IV, and X, suggesting a pleiotropic effect or tight linkage. Up to six QTL were identified for earliness. This understanding of the inheritance of yield and related traits can be used for marker aided selection. Variation in stipe, pileus, gills, and gill cavity has been observed in different isolates in our breeding program (Figure 4.9).

4.1.6.1.2 Cap Color

The majority of current button mushroom sales throughout the world are white mushrooms, while wild specimens are mainly brown with many gradations. Callac et al. (2005) reported that

Figure 4.9 Variability in stipe, pileus, gills and gill cavity in different accessions of button mushroom. (*See color plate section for the color representation of this figure.*)

all 21 isolates found under cypress had a brown cap color, while two out of 16 isolates from the open site had a cream cap color, and nine had light brown pilei (colors were measured after cultivation). Thus, genetic variability in this trait is available. Callac et al. (1998a) investigated the genetic basis of mushroom cap color by hybridizing a brown isolate and the white commercial hybrid U1 and postulated that a recessive allele at a single locus (PPC1) encodes the white pilei-pellis color. The PPC1 locus was mapped on chromosome VIII (Callac et al., 1998a). QTL analysis has made it possible to refine the inheritance of this trait. In addition to the major determinant PPC1 that explained 86% of the phenotypic variability, two minor loci were found on two other chromosomes, confirming the oligogenic control of this trait. These two additional loci contributed to the variability observed for the color gradation of the cap within the brown genotypes (Foulongne-Oriol et al., 2012a).

4.1.6.1.3 Bruising Sensitivity

Shelf life performance and susceptibility to discoloration (a consequence of the enzyme-catalyzed oxidation of phenols into quinones) after harvest affect the commercial value of mushrooms (Figure 4.10). Weijn et al. (2012) screened a collection of *A. bisporus* strains for their bruising sensitivity to analyze the phenotypic variation in susceptibility to discoloration after mechanical damage among strains. Studies by Gao et al. (2011) indicated that some brown wild strains showed less bruising sensitivity than white commercial lines. Genetic analysis of the trait by Gao et al. (2015) indicates that bruising sensitivity is a polygenic highly heritable trait (H^2: 0.88–0.96) and there is a significant interaction between genotypes and tester lines, and genotypes and flushes. Eastwood et al. (2001) identified 20 genes with increased expression levels by comparing 2-day post-harvest mushrooms with freshly harvested mushrooms.

4.1.6.1.4 Disease Resistance

Disease resistance in *A. bisporus* has recently been reviewed (Berendsen et al., 2010; Largeteau and Savoie, 2010), with significant reports of work on susceptibility diversity in many cultivars and wild strains. There are reports of QTL resistance to bacterial blotch, *Pseudomonas tolaasii* (Moquet et al., 1999); QTL resistance to *Trichoderma* metabolites (Foulongne-Oriol et al., 2011c); QTL resistance to *Lecanicillium fungicola*, bubble symptom (Foulongne-Oriol et al., 2012b); QTL resistance to *L. fungicola*, spotted cap symptom (Foulongne-Oriol et al., 2012b). In one of the first QTL mapping studies in fungi, Moquet et al. (1999) described a major QTL that underlay the resistance to *P. tolaasii*. This QTL explained about 30% of the phenotypic variation, and was closely linked to cap color locus PPC1 (Moquet et al., 1999). Complex

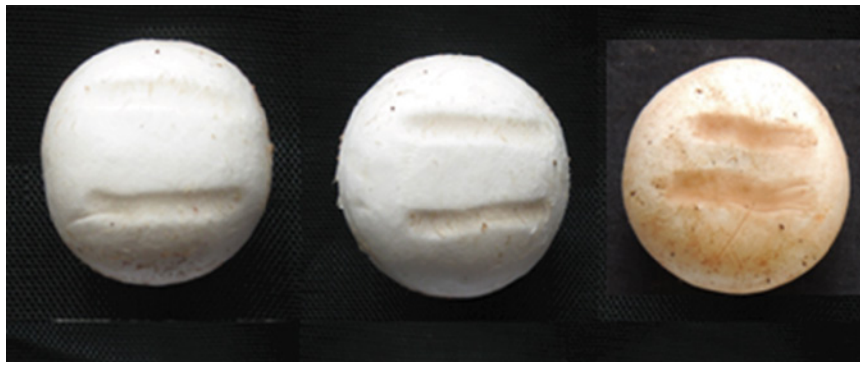

Figure 4.10 Variation in browning after 2 h. (*See color plate section for the color representation of this figure.*)

resistance to *L. fungicola* was also dissected through QTL mapping (Foulongne-Oriol et al., 2012b). Bubble and spotted mushroom, which are the two symptoms typifying dry-bubble disease during the successive steps of the infection, were analyzed separately. The QTLs involved in the expression of these two symptoms were detected as expected in distinct genomic regions.

4.1.6.1.5 Temperature Tolerance

A temperature of 16–19°C is needed during the fruiting period of *A. bisporus*. The development of *A. bisporus* cultivars able to fruit at higher temperature (FHT) represents a promising alternative to reduce energy costs during cultivation in hot countries as well as in temperate countries during the hot season. *A. bisporus* var. *burnettii* is able to fruit at 25°C. Understanding the biological mechanisms that underlie such a thermo-tolerance is a prerequisite to further development of breeding strains (Foulongne-Oriol et al., 2014).

4.1.7 Production and Identification of Homokaryons

To produce hybrids in the conventional manner, homokaryotic cultures have to be obtained by selecting monokaryotic basidiospores of the parental varieties of mushrooms. Such cultures of the opposite mating type will have to be fused to establish a heterokaryotic mycelium. However, homokaryons are very difficult to obtain by conventional basidiospore isolation from *A. bisporus* strains because of their secondarily homothallic life cycle (Raper and Raper, 1972). Some of the possible approaches to develop/identify homokaryotic cultures are described here.

4.1.7.1 Use of Non-Fertility as a Marker in Single Spore Isolates

This was the first successful approach used in mushroom hybridization. This method of isolating single spores and evaluating all isolates for fruiting is a time-consuming method. Despite this it is still used by most mushroom breeders because of its simplicity and reliability. It does not require much facility and is applicable in all cases. Spore prints are obtained using a standard procedure, and plated after repeated dilutions so as to get around 10 spores per plate. The spores are observed under a microscope and marked spores are picked after germination to develop single spore isolates. The next step is making spawn of all these cultures and evaluating these for fruiting ability. Those that do not fruit, that is, are non-fertile, are treated as homokaryotic.

4.1.7.2 Micromanipulation

In the initial stages when Elliot and other scientists were in the process of establishing type of mating system in *A. bisporus*, the sure way to demonstrate the type of spores produced on a basidium was to pick this using micromanipulation. For breeding purposes this approach is cumbersome even though most reliable and may also not need subsequent testing for fruiting to establish that the culture is homokaryotic.

4.1.7.3 Growth Rate and Downward Linear Growth on Compost

In general, homokaryotic cultures show poor growth in medium, though there can be wide variation in growth within non-fertile homokaryons. The downward linear growth of single spore isolates on compost is a more reliable method to segregate potential non-fertile isolates. Based on the study of 95 isolates that were evaluated for their downward linear growth on compost *in vitro,* selection criteria have been devised for preliminary identification of non-fertile isolates and rejection of likely poor yielding fertile isolates. Out of these 26 had very slow growth (3–7 mm in 3 weeks) and all of these turned out to be non-fertile. The remaining 69

isolates were fertile implying that initial classification of SSIs into fertile and non-fertile groups can be done on the basis of *in vitro* downward linear growth (Singh and Kamal, 2012).

4.1.7.4 Environmental Factors Enhancing Tetrasporic Basidia

The proportion of n-spored basidia depends not only on genetic factors but also on environmental conditions (Kerrigan and Ross, 1987). There are reports in the literature suggesting that basidia in the later stage of cropping and under higher temperatures and stress may produce more tri or tetrasporic basidia. In nature, such variations may be a mechanism of survival and propagation and there can be minor genes affecting the BSN gene, as dominance of this gene is at times reported to be incomplete. For practical purposes one may tend to collect mushrooms from later flushes. The gains, however, may not be significant and it may be more appropriate to select good quality fruit bodies from the early stage.

4.1.7.5 Use of the BSN Gene to Promote Production of Homokaryons

A. bisporus var. *burnettii*, known only in the population of the Sonoran Desert of California, is completely interfertile with the var. *bisporus*. Inter-varietal hybrids (var. *bisporus* × var. *burnettii*) have a predominantly heterothallic life cycle, because of the dominance (sometimes incomplete) of the tetrasporic allele (Bsn-t) at the BSN locus. Such hybrids make it possible to obtain large recombined homokaryotic progeny useful for breeding work, performing genetic maps, and studying the inheritance of traits of interest. The variety *A. bisporus* var. *eurotetrasporus* is also interfertile with both var. *bisporus* and var. *burnettii*, and most of the basidia of such hybrids are tetrasporic. Tests for allelism at BSN showed that both var. *eurotetrasporus* and var. *burnettii* bear similar dominant Bsn-t alleles (Callac et al., 1998b). *A. bisporus* var. *eurotetrasporus* represents a source of Bsn-t alleles useful for breeding work as does the var. *burnettii*, but also its haploid fruiting ability is a tool for studying development of the sporophores and the genetics of traits of interest (see Savoie et al., 2013).

4.1.7.6 Molecular Methods – RAPD, ISSR, and So On

A. bisporus mycelia where homo- and heterokaryotic mycelia cannot be easily differentiated as both are multinucleate and the latter shows no clamp connections. It is desirable to select molecular markers for breeding programs. Heterokaryons are likely to have various polymorphic loci whereas homokaryons will have only one allele. Such polymorphism by considering the number of loci can help in differentiating the homo- from heterokaryotic mycelia. There are various reports in this regard (Islam and Yinbing, 2010, 2011).

The availability of isozyme, RFLP, and RAPD markers has enabled routine analysis and isolation of homokaryons (Figure 4.11). Other uses are to identify and monitor crosses between homokaryotic isolates (Castle et al., 1987; Horgen et al., 1991). In our studies, we have found clear differences in fertile and non-fertile isolates (Annual Reports, DMR 2010–2014) using RAPD and ISSR markers (Figures 4.12 and 4.14). Considering that there is only a single allele of each gene in homokaryons, greater diversity in these was expected (Figure 4.13: DMR, 2014).

4.1.7.7 Protoplast Methods to Develop Homokaryons

The two methods most commonly used in the lab to develop homokaryotic cultures are protoplast formation and spore germination. Mushroom scientists have used both techniques successfully for many years (for techniques: see Horgen et al. (1991) for protoplasts and Kerrigan et al. (1992) for spore germination). The hyphae that make up a mycelium consist of cylindrical cells with strong walls. To release protoplasts these are treated with enzymes. The action of the enzymes develops weak spots or holes in the cell wall through which the cytoplasm (the cell contents) exudes. More than one protoplast may come from one cell. Thus, the

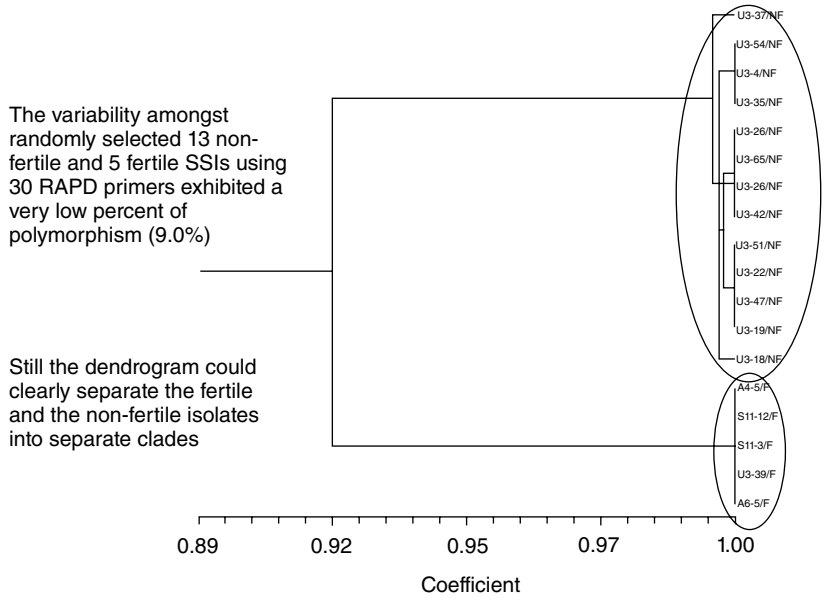

Figure 4.11 Dendrogram separating fertile and non-fertile isolates.

cell contents may be divided up into a number of compartments (Fritsche and Sonnenberg, 1988). The heterokaryon of *A. bisporus* contains an average of eight nuclei per cell (two types of nuclei with different compatibility factors). Some of the resulting protoplasts will have both types of nuclei, some only one. The first type, after regeneration, will produce a fertile heterokaryotic mycelium, the second type an infertile homokaryotic one. This method thus offers an alternative to the isolation of homokaryons via single spore cultures. In practice, almost 20% of the protoplasts obtained from the heterokaryon (U1) of *A. bisporus* form a homokaryotic mycelium. The homo- or heterokaryotic character of the mycelium can be determined rapidly by means of the iso-enzyme patterns. Likewise, other molecular markers can be used to identify homokaryons. We used ISSR markers to differentiate fertile and non-fertile isolates (DMR, 2014).

4.1.8 Hybridization: Intermating of Homokaryons/Heterokaryons

4.1.8.1 Intermating of Homokaryons

Homokaryotic mycelia are grown side by side and mycelium from the contact zone is picked (Figure 4.15). The cultures again need to be tested for fruiting to check the success of hybridization. Hybrids will be formed only when compatible mycelia are present in a petriplate. Some researchers propose the use of chemicals like nicotinic acid to promote fusion. Usually this may not be necessary.

4.1.8.2 Hybridization using Resistance Markers

Generally, a resistance marker is governed by insensitivity to an otherwise toxic compound. In this, a strain resistant to a fungicide can be intermated with another strain resistant to another fungicide and their hybrid can be identified by growing the intermated culture on both fungicides. At the Institute of Horticultural Research, UK, fungicides like benomyl and carboxin were used as the toxic substance and a group of mutants resistant to these compounds was

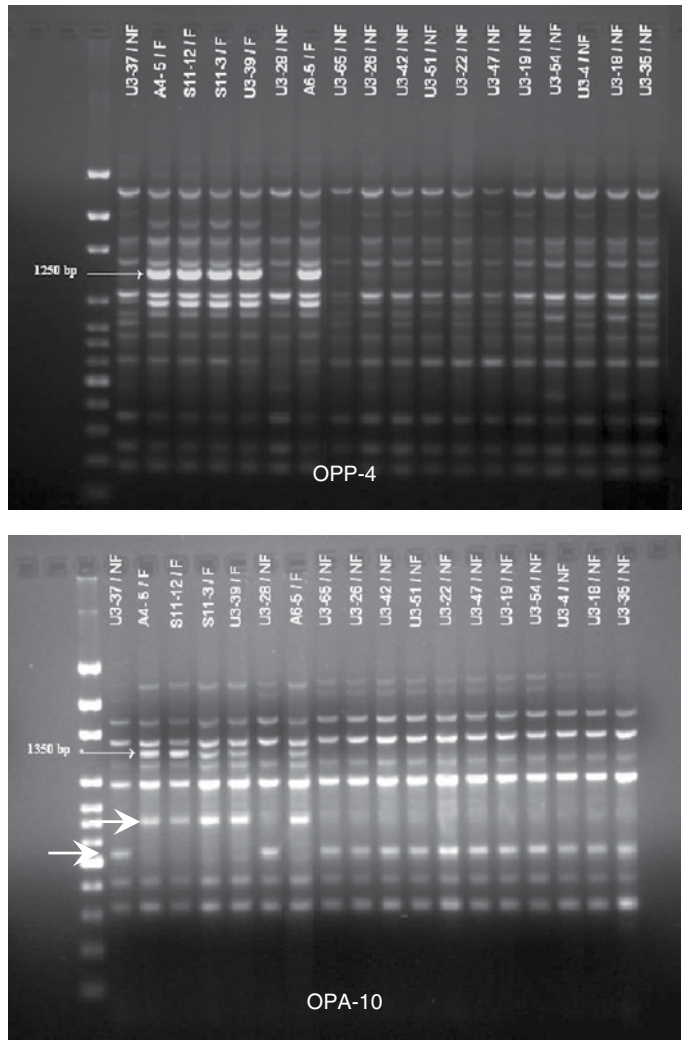

Figure 4.12 RAPD of fertile and non-fertile isolates of *A. bisporus*.

tested (Elliott, 1979b, 1982; Elliott and Challen, 1983). Several fungicide resistant mutants of *A. bisporus* were obtained. In addition, the range and type of resistance markers are also being studied (Challen et al., 1989). However, this approach has limitations and is more of a academic nature.

4.1.8.3 Hybridization using Auxotrophs as Markers

The auxotrophic strains have specific additional nutritional requirements for growth such as particular amino acids, vitamins, and so on that are not exhibited by the wild types. Therefore, hybrids can be formed by mating auxotrophs with different nutritional requirements so that each auxotroph complements the nutritional deficiency of the other in the hybrid formation (Elliott, 1987). Thus, this approach allows for confirmed isolation of the hybrid cells. Raper and coworkers (Raper et al., 1972) found the use of autotrophic strains to signal initial interaction and to trace the subsequent developments in identifying the progeny of actual crosses most

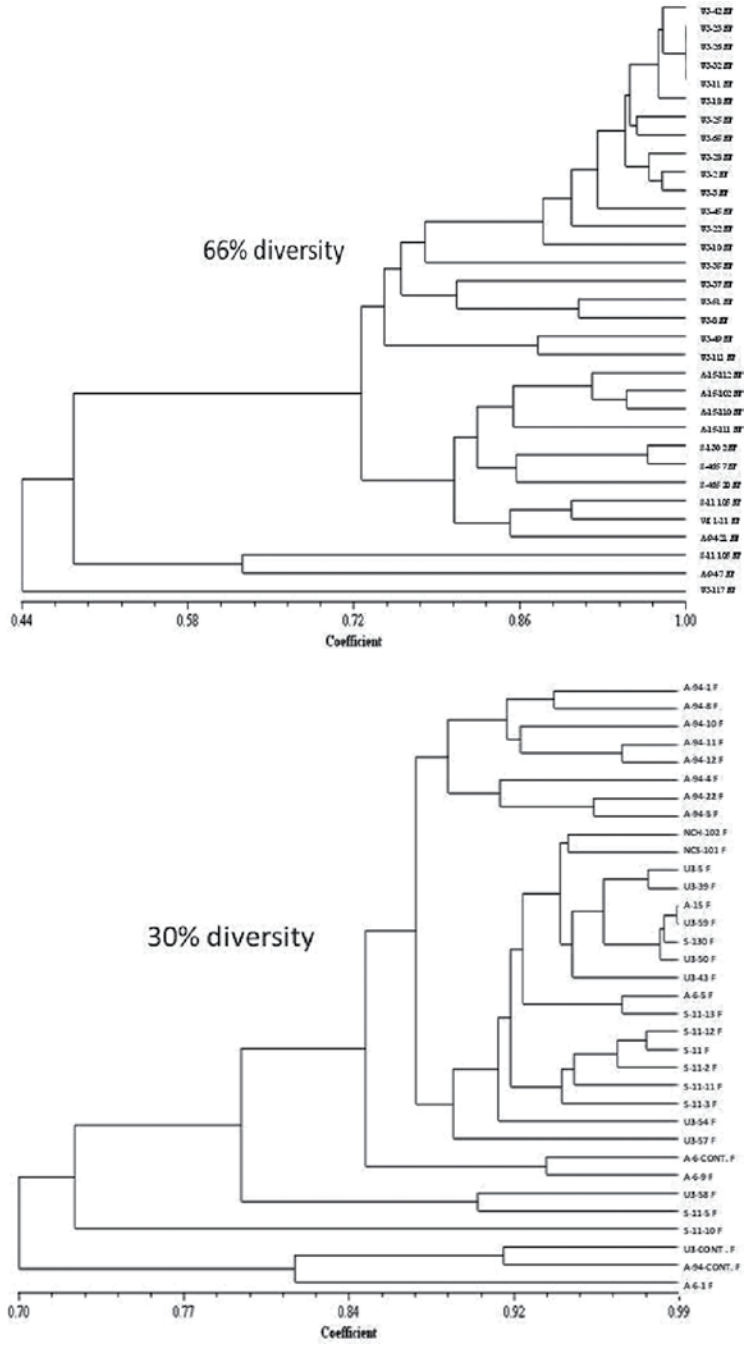

Figure 4.13 Diversity in homokaryons and heterokaryons.

useful. But the auxotrophs are not easily available and even if they occur cannot be detected due to the masking effect of the multikaryotic mycelial cells (Elliott, 1979; Wang, 1972). Hence, for practical purposes it may not be possible to obtain the desired auxotrophs for each strain

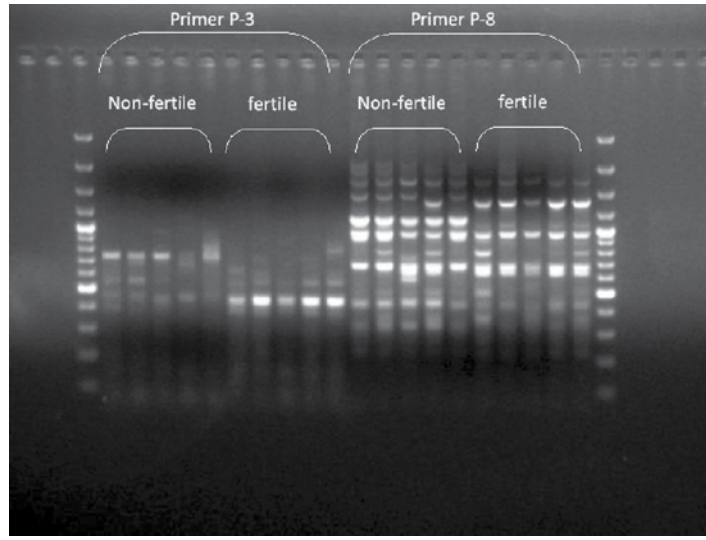

Figure 4.14 ISSR profile of five fertile and five non-fertile isolates using P-3 and P-8 primers.

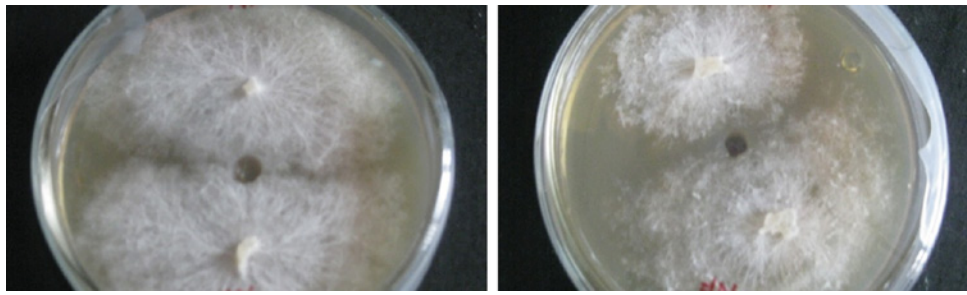

Figure 4.15 Intermating of non-fertile isolates.

even by induction with mutagenic agents, as the chances of mutation of desirable genes are also increased (Bhandal, 1990).

4.1.8.4 Somatic Hybridization/Protoplast Fusion

Protoplasts are cells, or fragments of cells, that no longer have a cell wall. The isolation of protoplasts is carried out in a medium with a high osmotic pressure. This prevents them taking up unlimited quantities of water and swelling up until the plasma membrane bursts. Protoplasts can be fused using polyethylene glycol or through electroporation (Fritsche and Sonnenberg, 1988).

The fusion of protoplasts was previously carried out using various chemicals such as calcium chloride or polyethylene glycol, where the yield of the hybrid cells was low. Use of electric pulsation (electrofusion) for protoplast fusion has resulted in a high yield of the hybrid cells. The protoplast fusion technique can be applied to isolate the homokaryons of a heterokaryon for further breeding as described earlier. Castle and coworkers (1987) have reported 10% regeneration of homokaryons by using protoplast fusion techniques, while Fritsche and Sonnenberg (1988) have obtained about 20% homokaryons from the heterokaryon of *A. bisporus* (strain U1) determined by means of isozyme patterns.

4.1.8.5 Di-Mon Mating

The two main ways of outcrossing are crosses between compatible homokaryons (heterothallic life cycle) and crosses between homokaryons and heterokaryons. The latter process, also called the "Buller phenomenon" (Buller, 1931) was first reported by Raper et al. (1972) in *A. bisporus*. During the Buller phenomenon (Buller, 1931), anastomosis between a homokaryon and a heterokaryon produces a hybrid heterokaryon that receives the nucleus of the homokaryon and one of the two nuclei of the heterokaryon (which must be sexually compatible with the nucleus of the homokaryon). This process occurs in many Basidiomycetes and is generally accompanied by nuclear migration from the heterokaryon through the homokaryon, which becomes heterokaryotic. The Buller phenomenon occurs in *A. bisporus*, but so far there is no evidence for nuclear migration in this species (Raper et al., 1972).

In different experiments, Callac et al. (2003, 2006, 2008) inoculated a standard substrate for *A. bisporus* cultivation simultaneously with homokaryotic mycelium from one parent and spores from a second parent. Culture trays have consistently produced numerous sporophores that could theoretically have resulted from five different reproductive modes (pseudohomothallism, selfing or outcrossing via heterothallism, and selfing or outcrossing via the Buller phenomenon, Figure 4.16). However, genotype analysis showed that all or almost all the sporophores consistently resulted from outcrossing between the inoculated homokaryon and the inoculated heterokaryotic spores (or mycelia that grew from them), that is, via the Buller phenomenon. While evaluating single spore isolates for fertility, we observed that even non-fertile isolates produced a few fruit bodies after a few weeks and this may be the result of random spores falling on these bags and natural intermating (Singh and Kamal, 2011b). Thus, homokaryons can be used in the same way breeders use male sterile lines to produce hybrids in higher plants. The drawback will be that we won't have complete knowledge of both the parents and selections will be based on the quality and performance of the random hybrids.

With this new method, any wild strain that can yield a spore print can be used as a parent. This method used for hybridization and selection also has a disadvantage. Since crosses occur via the Buller phenomenon without gametic selection, undesirable parental alleles can be transmitted to the hybrids. Thus, the best use of this method probably is in the early generations of selection. One solution to this problem is to inoculate the grain spawn with a homokaryon that carries the *Bsn-t* allele, which results in a high percentage of tetrasporic basidia (Callac et al., 1998a,b; Imbernon et al., 1996). All the hybrid sporocarps would then carry this dominant allele and should produce primarily homokaryotic spores that can be used in the next generations of selection.

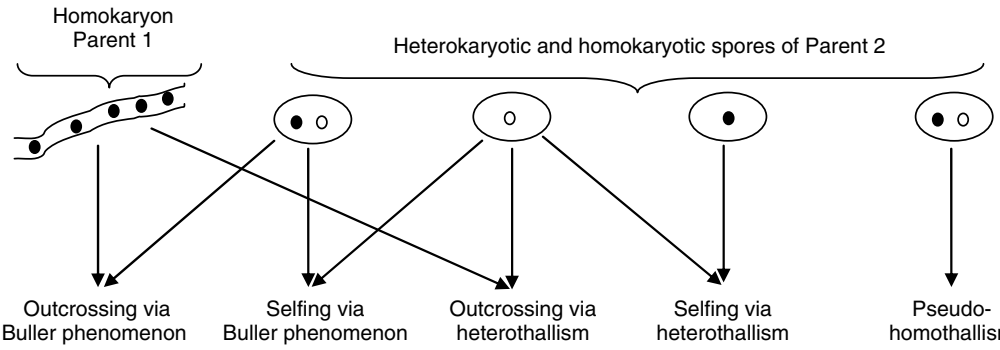

Figure 4.16 Five possible matings on mixing homokaryotic mycelium with spore print of other parental strain.

4.1.8.6 Identification of Hybrids using Isoenzyme/Molecular Markers

Isoenzymes are the multiple molecular forms of an enzyme which are the products of individual genes. These isoenzymes can be characterized on a gel (gel electrophoresis) in the form of bands and the banding pattern of different groups of enzymes can be used for fingerprinting a particular strain. Strains having different banding patterns can be used for crossing and the hybrid recognized on the basis of gel electrophoresis. This novel approach was developed in the USA to identify the hybrids in *A. bisporus* (May and Royse, 1982; Royse and May, 1982a,b; Royse et al., 1982). Enzyme markers have an advantage over auxotrophs that the former are naturally occurring and there is no limit to the number of crosses that can be made. The hybrids can be identified using biochemical and molecular markers like RAPD, RFLP, ISSR, and so on. We use retro-element based markers that have shown very high resolution between the parents and hybrids. Thus, the number of molecular approaches can be used for confirmation of hybridization in an *Agaricus* breeding program. The final confirmation will be through field trials.

4.1.9 Genetic Manipulation: Transformation

The conventional breeding methods involve the use of sexual interaction between two parents possessing the desirable characteristics through crossing. The techniques of genetic manipulation allow the transfer of desirable characteristic into a strain without sexual interaction. This technique involves the characterization and cloning of genes and their transfer within or between species through vectors or electroporation. Gene transformation has been carried out in mushrooms like *Coprinus* spp. and *Schizophyllum commune.* The two most important techniques of genetic manipulation are somatic hybridization and genetic transformation. Somatic hybridization as described above, involves the fusion of two different types of cells. The direct gene transfer system is a much more specific method whereby one or more genes coded for a desirable characteristic can be isolated and transferred by genetic manipulation. The latter has an added advantage of overcoming the crossing barriers especially between strains which are not related.

Transformation of yeast and filamentous (ascomycetous) fungi has now been common practice for many years. Hinnen et al. (1978) were the first to report the complementation of a haploid *Saccharomyces cerevisiae* LEU2 strain. Meanwhile, many yeasts and filamentous fungi appeared transformable with both auxotrophic and dominant markers (for reviews see, e.g., Gurr et al., 1987; Fincham, 1989; Reiser et al., 1990; Van den Hondel and Punt, 1991). Homobasidiomycetes, the class to which many mushrooms belong, including well-known edible mushrooms like *A. bisporus*, *Pleurotus ostreatus* (oyster mushroom), and *Lentinula edodes* (shiitake), generally demonstrated a higher reluctance to genetic transformation. The first successful transformations on homobasidiomycetes were carried out using a tryptophan-auxotrophic (trpC) mutant of the wood rotting fungus *Schizophyllum commune*, the homologous TRPC gene and polyethylene glycol-mediated DNA entry into protoplasts (Munäoz-Rivas et al., 1986). The *A. bisporus* transformation system is based on selection with the *Escherichia coli* hygromycin B resistance (hpt) gene, introduced via electroporation into protoplasts (Van de Rhee et al., 1996). The primary scientific application of the *A. bisporus* transformation system will be the silencing of genes in order to find out their specific function. An alternative way to introduce donor DNA into a vast number of organisms, including many plant and fungal species, and even intact tissues, is particle bombardment. This technique is based on gas-driven bombardment with tungsten or gold particles coated with the donor DNA, thus penetrating the recipient tissue.

4.1.10 Selection

4.1.10.1 Direct Selection

Fritsche adopted a five-step selection procedure that involved evaluation of each isolate in (1) one or two trays with 2.5 kg compost and screening for quality, (2) two trays of 1.3 m^2 and screening of morphological traits over flushes, (3) four trays of 1.3 m^2 and initial screening for yield, (4) final selection on eight trays of 1.3 m^2 and also evaluation for storage and processing parameters, and (5) final evaluation on five trays of 6 m^2 for yield and selection to be done by an expert committee also. The evaluation methods in terms of nomenclature may vary and be named differently, for example, as initial evaluation trials, advance evaluation trials, field evaluation trials, and so on, but the essence remains the same that we screen first for quality and then quantity.

4.1.10.2 Selection by Rejection

Our studies on downward linear growth of 69 fertile cultures on compost *in vitro* showed that the yield of these cultures was non-significantly correlated with radial growth on malt extract agar but had highly significant positive correlation (r = 0.727) with downward linear growth (Singh and Kamal, 2012). This suggests that it may be possible to reject likely poor yielding cultures on the basis of their downward growth on compost *in vitro* and the selected ones can be evaluated for yield and other traits (Figure 4.17). This approach is highly useful when we may be handling large numbers of single spore isolates and may be interested in selecting few single spore isolates.

As a breeder one may be tempted to directly evaluate all hybrids. Even if a hybrid may be yielding a bit less but have desirable quality, it may be worthwhile to retain it and isolate suitable culture from its single spore isolates. Hence, the more appropriate approach of selection by rejection may be based on fruiting body quality, that is, size, shape, color, firmness, scaliness, length of stipe, color of gills, distance and size of gill cavity, thickness of pileus, response to bruising, and so on. All this will require initial evaluation trials of all or selected cultures on a very small scale where fruit bodies can be evaluated for such parameters. Any strain even if a good yielder but not having desirable quality will have no place in the market. In subsequent evaluations, the pattern of flushes, yield, response to particular traits like canning, or even nutritional and medicinal traits like ergosterol or eritadenine content, and so on, can be done.

4.1.10.3 Marker-Assisted Selection (MAS)

MAS is an approach to select the genotype by identifying the marker(s) linked to the trait rather than to select on the phenotype (Collard and Mackill, 2008; Hospital, 2009). Since most of the important traits in edible mushrooms can only be observed at fruiting stage, early selection with molecular markers allows an accurate screening of desirable offspring without the cultivation step. Molecular markers in *A. bisporus* have already been used for homokaryotic spore isolation (Kerrigan, 1992), cap color selection (Loftus et al., 2000; Foulongne-Oriol et al., 2011b), or mating-type compatibility design (Sonnenberg et al., 2005). A first marker-assisted backcrossing program for a polygenic trait introgression was mentioned in Sonnenberg et al. (2005).

The next step after selection by rejection and marker aided selection will be evaluation in controlled conditions. Despite being an indoor crop, the performance can vary between research organization and commercial units. Hence it is appropriate to undertake final evaluation at a commercial unit.

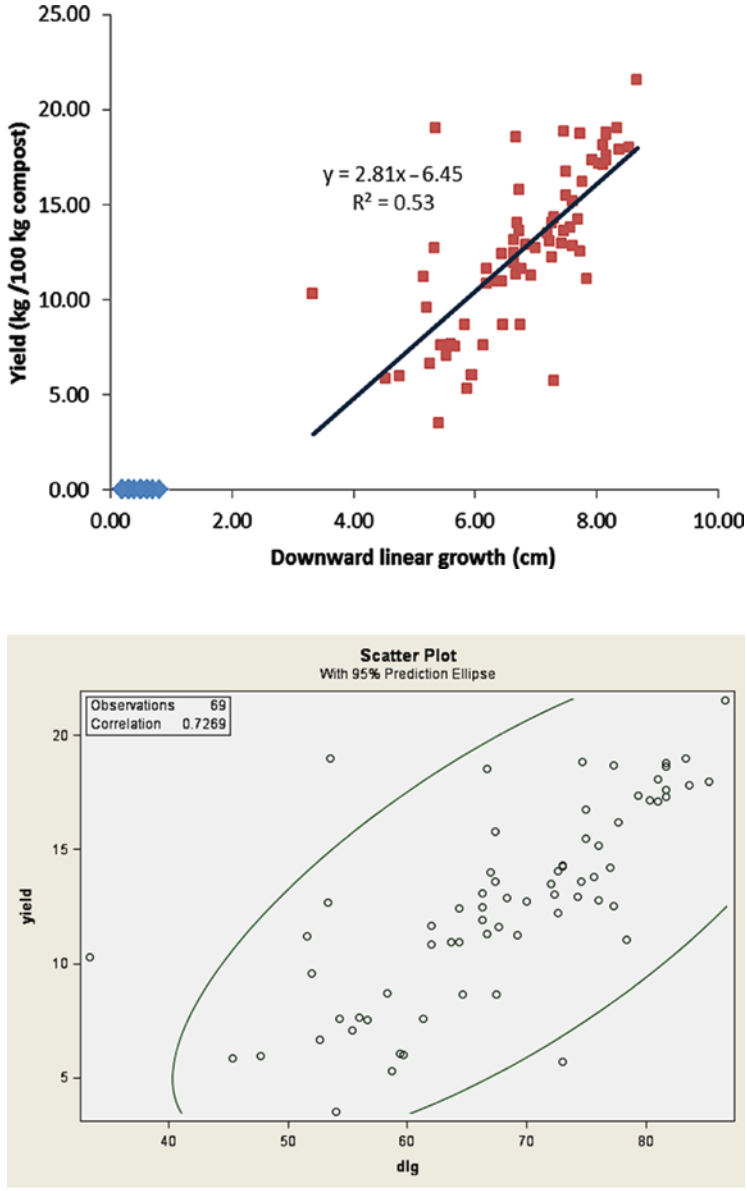

Figure 4.17 Above: yield and downward linear growth of non-fertile (left) and fertile (right) isolates, and Below: scatter plot of yield versus downward linear growth of 69 SSIs of *A. bisporus*.

4.2 Oyster Mushroom (*Pleurotus* Species)

4.2.1 Introduction

In the genus *Pleurotus* more species have been reported as cultivated than in any other genera of agarics. It was first cultivated experimentally on tree stumps and sterilized straw by Falck (1917) and on sawdust cereal mixture by Kaufert (1935). Despite its late introduction and domestication when compared with that of the button mushroom, *Pleurotus* species have

become a well recognized part of the world mushroom industry. From 1997 to 2010, global production of *Pleurotus* spp. increased from 0.876 million ton to 6.288 mt (618%). China was responsible for most of the production increase and accounted for over 85% of the world's total output in 2010. Approximately 25% of China's mushroom production in 2010 was from two species of *Pleurotus*: *P. ostreatus* and *P. cornucopiae*. In the last 5 years or so, however, substantial increases in production of *P. eryngii* and *P. nebrodensis* have occurred. The *Pleurotus* species at present constitute 27% of world mushroom production (Royse, 2014). *Pleurotus* can grow on a wide variety of lignocellulosic substrates, namely, hardwoods, sawdust, paper, paper sledges, cereal straws, corn and corn cobs, coffee residues such as coffee grounds, hulls, stalks, and leaves, banana fronds, and waste cotton (Ralph, 2005). Its desirable attributes, such as high saprophytic colonization ability on a wide variety of substrates, rapid mycelial growth, simple and cheap cultivation techniques, and easy post-harvest storage have contributed to its popularity.

4.2.2 Reproductive Biology

In *Pleurotus* spp. basidiospores germinate to produce monokaryotic hyphae. Anastomosis of two compatible monokaryotic hyphae results in dikaryotic mycelia where each cell has two nuclei from an opposite compatible mating type. There is a unique method of clamp connections to ensure that each cell has two nuclei of opposite mating type. Fusion between two nuclei occurs only in the basidium. Immediately after fusion, meiotic division occurs, and this results in formation of four basidiospores. The variability created by mutations expresses its potential by intermating of different genotypes where new combinations arise due to genetic assortment. In most of the organisms the outbreeding is achieved by sexual differentiation. In Basidiomycetous fungi it has been achieved by evolution of incompatibility factors and the degree of outbreeding is dependent upon the number and structure of incompatibility factors. Determination of mechanism of incompatibility essentially involves isolation of single spores and intermating of monokaryotic hyphae that fuse to give rise to dikaryotic mycelium which can be differentiated by the presence of clamp connections (Eger, 1978). Vandendries (1933) was the first to detect bifactorial heterothallism in *P. ostreatus* by the analysis of clamp formation. This was later confirmed by various researchers (Terakawa, 1960; Engenio and Anderson, 1968). Kaufert (1936) has described *P. corticatus* (suggested as a common identity for *P. cystidiosus* by Jong and Peng (1975)) as similar to *P. ostreatus* with the exception that the dikaryon as well as homokaryon have an asexual cycle. Bifactorial heterothallism has also been reported in *P. sajor-caju* (Roxon and Jong, 1977; Thakur and Bhandal, 1993), *P. flabellatus* (Chandershekhar et al., 1981), *P. sapidus* (Thakur and Bhandal, 1993), and many other *Pleurotus* species. As we know, bifactoriality and allelic polymorphism promote outcrossing (Hsueh et al., 2008; Heitman et al., 2013) and the chance of being compatible for any two haploid products from the same diploid parent is 25% in a bifactorial system with completely unlinked mating-type loci (Hsueh et al., 2008).

Thus, in *Pleurotus* species inbreeding is avoided by an incompatibility system determined by two loci each with multiple alleles (A and B mating-type loci). Multiple allelism for both incompatibility factors was demonstrated by Engenio and Anderson (1968). In *P. ostreatus* more than 30 different A-factors and a comparable number of different B-factors have been found (Vandendries, 1933; Terakawa, 1960; Engenio and Anderson, 1968). Strains originating from different parts of the world and growing on different substrates display 100% compatibility in crossing experiments (Bresinsky et al., 1987). By analysis of the segregation pattern of a single gene mutant for alleles of incompatibility and fruiting body color, Arita (1974) gave the genetic proof of the haploid homokaryotic nature of basidiospores in *P. ostreatus*.

Kothe (2001) explained the role of the two loci A and B. Genes of A loci lead to production of transcriptional factors, a homeodomain protein. During the mating of two different mating-type cells, heterodimerization of two proteins of different allelic specification occurs and leads to formation of active transcriptional factor. Factor A is known to control nuclear pairing, clamp cell formation, coordinate cell division, and clamp cell septation. The products of B loci, a pheromone, and receptor system, lead to nuclear migration, septa dissolution, and clamp cell fusion.

The genetic structure of both the mating types is complex. The factor A gene complex consists of a central motif of two genes (coding for the two protein types present in heterodimer) transcribed in divergent directions that appears duplicated one to three times in the different A mating types and species. In *P. ostreatus*, locus A behaves as a single one, whereas locus B is a complex of two genes, *matBα* and *matBβ,* based on identification of molecular markers linked to the *mat B* locus (Larraya et al., 2001). A similar type of mating system was reported by James et al. (2004) in *P. djamor*.

Larraya et al. (1999a,b) analyzed the genomic constitution of *P. ostreatus* by using pulsed-gel electrophoresis optimized for the separation of its chromosomes and determined that it contains 11 pairs of chromosomes with sizes ranging from 1.4 to 4.7 Mbp with total size of 35.1 Mbp. Their study also revealed the occurrence of relevant chromosome length polymorphisms (CLPs) between homologous chromosomes in each of the two nuclei occurring in the dikaryon. CLPs are common in fungal genomes and have been related to variations in the copy number of sequences such as rDNA, subtelomeric repeats, and in the result of meiotic and mitotic recombination process.

4.2.3 Germplasm

The first step in any improvement program is collection of natural variability. The proper understanding and exploitation of the natural variability can give good return in the initial stages of any genetic improvement program. But in mushrooms the desired amount of emphasis has not been given in this direction even though many species have been cultivated experimentally (Seth, 1986). Generally, the few fruit bodies picked up by a worker form the basis of a strain which gets distributed to various parts of the world. In the absence of diverse material, the gains from genetic manipulations become limited. Thus, it is important to collect and catalog large number of strains from different hosts and locations. With increasing deforestation, edible fungi are also being affected and we may be losing valuable germplasm. India, like many other countries, has a rich diversity of *Pleurotus* species (Figure 4.18). At the Directorate of Mushroom Research, Solan (India) we have been collecting germplasm through regular explorations and have 360 accessions of *Pleurotus* species in the gene bank (Upadhyay et al., 2016).

4.2.4 Genetic Variability

Pleurotus belongs to the family Tricholomataceae and has about 40 well recognized species, out of which 12 species are cultivated in different part of India (Ahmed et al., 2009). The well-known species of *Pleurotus* include *P. cornucopiae*, *P. pulmonarius, P. populinus, P. ostreatus, P. cystidiosus*, *P. djamor*, *P. eryngii*, *P. calyptratus*, *P. dryinus*, *P. purpureo-olivaceus*, and *P. tuber-regium*. Variations in sporophore color, margins, shape, size, stipe length, and so on, can be observed in different species and within species. Many of these characteristics are affected by environmental factors like light, temperature, carbon dioxide, humidity, and so on. However, under controlled conditions, the morphological characters, yield, texture, flavor, and other traits are stable and can be compared within and between species. Genetic variability has been demonstrated in *Pleurotus* among the strains originating from different locations.

Figure 4.18 Naturally occurring *Pleurotus* species in forests of Maharashtra (India).

It has been demonstrated that ability to fruit in a wide range of temperatures is controlled by a single dominant gene (Bresinsky et al., 1987; Li, 1980). It may be mentioned here that dikaryons and fruit bodies formed by outbreeding often shown higher vitality (Engenio and Anderson, 1968; Prillinger and Molitoris, 1979). In the absence of sufficient germplasm or desirable traits within a species, interspecific hybridization may be useful for developing new variability.

May and Royse (1988) collected 60 cultures of different *Pleurotus* species from worldwide sources and studied interspecific allozyme variation. There are a number of studies where intra- and interspecific variations in *Pleurotus* species at DNA level have been studied using various markers like RAPD (Fonseca et al., 2008; Kavousi et al., 2008), RFLP (Bao et al., 2004), AFLP (Jusuf, 2010), ISSR (Wang et al., 2012), and so on. A high degree of diversity can be detected using DNA based markers. This variability is good to understand the extent of variation and evolutionary distances, but may not be a reflection of variation in economic traits. A major part of the variation here is in non-functional DNA. Contrary to this variation at enzymatic or protein level is the reflection of functional genes. However, for breeding purposes our interest may be in a few genes or gene complexes determining color, texture, yield, flavor, disease resistance, or other economic traits.

Nuclear-encoded ribosomal RNA genes (rDNA) have been the primary focus of investigation for new taxonomic approaches in fungal molecular systematics. The genes for the small

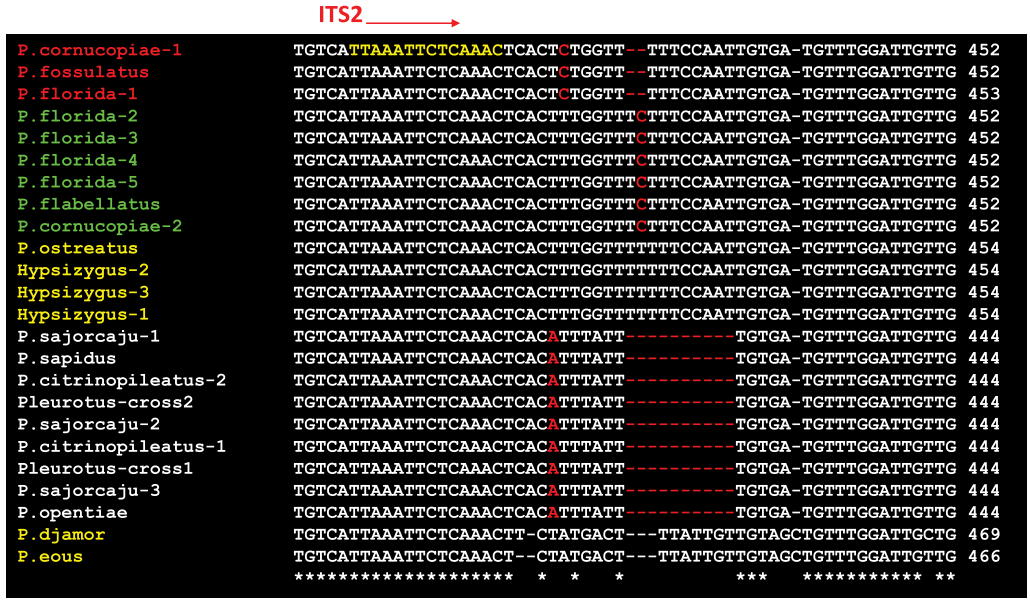

Figure 4.19 Analysis of ITS2 sequences in *Pleurotus* species available at DMR Solan.

subunit (18S), 5.8S and large subunit (25–28S) are separated by one or more intergenic spacer (IGS) regions. The coding regions of the 18S, 5.8S, and 28S nuclear rDNA genes are highly conserved among fungi. They show little sequence divergence between closely related species and are useful for phylogenetic studies among distantly related organisms (Berbee and Taylor, 1992; Swann and Taylor, 1993, 1995; Binder and Hibbett, 2002).

The conserved regions are separated by internal transcribed spacers, ITS1 and ITS2, which show higher rates of divergence. ITS1 is located between the 5.8S gene and the 28S gene (Guzeldag and Colak, 2007). The ITS2 sequences in germplasm available at DMR Solan have been analyzed by Dr MC Yadav (NRCM, 2006) (Figure 4.19).

4.2.5 Genetic Parameters: Heterosis, Heritability, Combing Ability

Genetically improved strains not only increase the quality of cultivated mushrooms, but also reduce the costs of cultivation. They can also increase farmers' revenue in the short term (Avin et al., 2012). In crop plants, there is lot of emphasis on parameters like heritability and combing ability. Understanding of these parameters in the long run can help to choose the most suitable breeding scheme for improvement of a particular trait. The crossing programs in fungi are seldom viewed from this angle. Phenotypic variance is the sum total of genotypic variance, environmental variance, and their interaction ($V_P = V_G + V_E + V_{GE}$). Genotypic variance can be partitioned into additive and dominance components ($V_G = V_A + V_D$). Avin et al. (2016) made an effort to work out components of variance, correlations, additive and dominance gene effects, heterosis, narrow- and broad-sense heritability, and general and specific combing ability of various yield traits in *P. pulmonarius* after attempting diallel crosses and generated superior hybrids. The newly generated hybrids were identified by both morphological and molecular fingerprinting (PCR–RFLP). In this study the proportion of environmental effects as well as the interaction of the environment genotype were negligible, while the proportion of genotypic variance was very substantial. The genetic variance components and heritability analysis

indicated that the proportion of dominance variance among the overall genetic variance was higher than additive variance. These parameters indicate that hybridization and simple selection can be effective in this species. The simplest approach for improvement in any trait is the selection of desirable types from the existing variability, but for higher gains it is desirable to choose a planned hybridization program.

4.2.6 Hybridization

A tetrapolar mating system with four-spored basidia and clamp connections in this genus makes attempts at strain improvement easier (Terakawa, 1960; Engenio and Anderson, 1968; Larraya et al., 1999a,b). The two mating factors with their mating-type loci are used as markers for breeding and have been incorporated in a chromosome mapping investigation (Larraya et al., 2000; Ramírez et al., 2000). The important characters within the gene pool of a particular edible mushroom species are always limited and thus such necessary characters need to be acquired from some other strains/species/genera across the genetic exchange boundary barrier of that particular organism. With this understanding in mind, researchers have developed different techniques of hybrid production to improve the different edible mushroom strains.

4.2.6.1 Conventional Breeding

Crosses by intermating single spore isolates within strain, between strains of same species and strains of different species are possible in *Pleurotus.* This conventional method of breeding has been used by various scientists to improve the yield and quality parameters. Pandey and Tewari (2004) developed a low spore producing strain of *P. florida* by cross hybridization. Gharehaghaji et al. (2007) developed 27 hybrids by intermating five strains of *P. ostreatus*.

4.2.6.1.1 Intra-Strain Hybridization

Gupta et al. (2011) isolated 182 single basidiospores from mushroom bodies of *P. sajor-caju, P. florida, P. eous*, and one wild relative of *Pleurotus* called *Hypsizygus ulmarius*, classified these into mating types and undertook intra-strain matings in the five species. We also intermated monokaryons of the same strain in *P. sajor-caju* and *P. sapidus* and the cultures so produced did not yield any strain that was able to yield above the parental strain (Thakur and Bhandal, 1993).

4.2.6.1.2 Inter-Strain/Intra-Specific Hybridization

In *Pleurotus* a high amount of variability is generated by intermating of different strains (Bresinsky et al., 1987). The inter-strain hybridization will involve isolation of single spores and intermating of such monokaryotic isolates from different strains. Due to the existence of multiple allelism in incompatibility factors, monokaryotic mycelia of *P. ostreatus* derived from different fruit bodies (representing different strains collected from different locations or hosts) intermated at a 100% rate compared to an inbreeding capacity of only 25% (Bresinsky et al., 1987). It is likely that the same will be true of strains in other *Pleurotus* species. As mentioned previously, in *Pleurotus* species, the monokaryotic hyphae lack clamp connections and dikaryotic mycelium can be identified by the presence of clamp connections. The procedure of single spore isolation and their intermating can be completed in the laboratory itself. The preliminary screening for fruiting body color, size, shape, texture, and so on can be done in small bags. It will, however, be necessary to have replicated trials (with appropriate randomization) while screening for yield. Screening of all the hybrids produced by intermating will require immense space and manpower. It is thus desirable to have selection criteria at early stages.

Wang and Anderson (1972) while genetically analyzing the sporocarp production in *P. sapidus* found a high correlation in rate of growth of dikaryon and their component monokaryons. Similarly, radial growth of *P. ostreatus* dikaryons was also found to depend on the growth of monokaryons (Bisko and Kosman, 1989). In both *P. ostreatus* (Eger, 1974) and *P. flabellatus* (Samsudin and Graham, 1984) correlation has been shown between monokaryotic fruiting and yield. Small pinheads that never develop into fruit bodies were also reported in 35% of the monokaryotic isolates of *P. sajor-caju* (Singh and Mehta, 1986).

In *Schizophyllum commune*, the strong influence of mating of F_1 monokaryons on dikaryon fruiting suggested that the same genes were involved in mono- and dikaryotic fruiting (Mattila, 1989). Monokaryotic fruiting has been reported in many Basidiomycetes and its genetic control has been worked out in some of these (Chang and Miles, 1989). A better understanding of genes involved in the fruiting of mono- and dikaryotic mycelia of different *Pleurotus* species may help in formulation of appropriate selection criteria. As the screening of monosporus isolates is easy and quick, selection criteria like mycelia growth rate, monokaryotic fruiting, etc. may help in saving time and effort in genetic improvement programs. Bisko and Khalodry (1989) have used a monospore selection method for obtaining high productive strains of *P. ostreatus,* the multiple indexes at monokaryotic and dikaryotic stages included mycelia growth rate, primordial formation, utilization of different nutrient sources, temperature dependence of mycelia growth and fruiting body formation, productivity, and biological value of fruiting bodies.

Kumara and Edirimanna (2009) intermated monospore cultures of two strains of *P. ostreatus* (Lanka Oyster and American Oyster) to produce 23 hybrids. Interspecific hybridization studies were carried out by Bahukhandi and Sharma (2002) between *P. sajor-caju, P. sapidus*, and *P. cornucopiae.* Goltapeh et al. (2007) attempted hybrid production of oyster mushroom *P. ostreatus* (Jacq: Fries) Kummer by intermating monokaryons from five strains collected from west Iran and produced 27 potential hybrids. Strain C5 of *P. eryngii* was developed by mono–mono crossing between monokaryotic strains derived from KNR2594 and KNR2610. Although the production yield of C5 was slightly lower than that of the strain KNR2594, the quality did not change and remained normal after a period of 59.0 d at 4°C (Kim et al., 2013). Im et al. (2013) developed hybrids in *P. eryngii* with a high yield and earliness of harvest trait and its sensory test.

4.2.6.1.3 Interspecific Hybridization

Different species can be crossed by intermating single spore isolates or by protoplast fusion. Details of the latter are given in the next section. *Pleurotus* species are widely distributed and can be broadly divided into two groups – low temperature strains fruiting below 15°C (Kuehner and Ramagnensi, 1974; Bresinsky et al., 1977; Hilber, 1977) and temperature tolerant strains which can fruit in a wide range up to 27°C (Engenio and Anderson, 1968; Anderson et al., 1973). For example, *P. ostreatus* and *P. columbines* are low temperature strains, while *P. pulmonarius, P. cornicopiae, P. sapidus, P. florida* are temperature tolerant. In the genus *Pleurotus* a number of species have been reported to intermate under laboratory conditions. *P. florida* has been shown to intermate with *P. ostreatus* (Eger et al., 1976; Bresinsky, 1987) and is regarded as a high temperature strain of *P. ostreatus. P. sapidus* has been reported to intermate with *P. ostreatus* (Eger et al., 1976, 1979). Three European *Pleurotus* species, namely *P. ostreatus, P. columbines*, and *P. pulmonarius* were shown to interbreed with *P. sapidus* (Manning et al., 1977); *P. columbines* was inter-breedable with *P. florida* (Bilber, 1978), *P. abalonus* and *P. cystidiosus* were interbreedable though confrontations between monokaryotic mycelia of both taxa resulted in a 50 or 100% compatibility compared to an expected 75 or 100% compatibility in the case of inter-strain crosses within a single species (Bresinsky

et al., 1987). The mating compatibility studies have demonstrated the existence of 11 discrete intersterility groups in *Pleurotus* (Zervakis and Balis, 1996).

The various interspecific interbreedability results have led to different opinions about species. The two populations may have actually developed isolation barriers in nature but are able to intermate under laboratory conditions. There can also be some cases of misidentification and collections categorized as separate species may be the same species. A collection may be categorized as different species because it may be showing different morphology on different hosts or under different locations, or morphological and physiological differences between two species may be so minute that it is sometimes difficult to distinguish them from each other in the field (Bresinsky et al., 1987). Irrespective of the inferences one may draw from intermating of different species, it is clear that such mating can be explored for broadening the genetic base of material. As in button mushrooms, many of the hybrids have been patented. For example, Patent CN 104717881 A was filed in Mar 18, 2009 for novel species produced by interspecies crossing among *Pleurotus* spp. varieties of *P. eryngii* Quél, which are not native to Japan and *P. ostreatus* Kummer, which is native to Japan.

4.2.6.3 Protoplast Fusion

Protoplast fusion is an important tool in strain improvement for bringing genetic recombination and developing hybrid strains in filamentous fungi. Protoplast fusion has been used to combine genes from different organisms to create strains with desired properties. These are powerful techniques for engineering of microbial strains for desirable industrial properties (Aswini et al., 2014). Protoplast fusion continues to be an exciting area of research in modern biotechnology (Carrillo et al., 2011). Protoplast fusion has been used as a method to create mushroom hybrids especially when conventional methods are not successful. It can be performed intraspecifically (Kiguchi and Yanagi, 1985; Toyomatsu and Mori, 1987), interspecifically (Takehara et al., 1993; Matsumoto et al., 1997; Dhitaphichit and Pornsuriya, 2005; Selvakumar et al., 2015), intergenerically (Eguchi et al., 1993; Zhao and Chang, 1993, 1996), and even interheterogenerically (Toyomatsu and Mori, 1987; Eguchi and Higaki, 1995).

Protoplast fusion is an efficient method to generate novel mushrooms even from two distantly related species (Lee et al., 2011). The step by step procedure has been described by Sodhi and Jaswal (2016). It essentially involves protoplast isolation by washing the mycelia with osmotic stabilizer and treating it with cell wall degrading enzymes under isotonic environment. The released protoplasts are separated, purified, washed in osmotic stabilizer, and then used for fusion and regeneration. The osmotic stabilizers and media used for protoplast regeneration can significantly influence the rate of protoplast regeneration. The type and concentration of osmotic stabilizers and media used for optimal protoplast regeneration differs for different fungal species. The protoplast fusion can occur spontaneously or it can be induced either mechanically, using chemical inducing agents, or with electric shocks. Two fusion methods, namely, chemically-induced fusion (commonly PEG-induced) and electrofusion have been most widely used (Peberdy, 1991). Induced protoplast fusion overcomes vegetative incompatibility and produces hybrids with combined properties of both parents.

Since the isolation of protoplasts in *Schizoplyllum commune* by de Vries and Wessels (1972) some reports have appeared on the isolation and regeneration of cultivated basidiomycetes like *Agaricus bisporus* (Fritsche and Sonnenberg, 1988) and *Pleurotus sajor-caju* (Liao et al., 1989). Magae et al. (1985) demonstrated the ability to produce fruit bodies from protoplasts of *P. ostreatus*. Interspecific protoplast fusion has been achieved between *P. salmoneo-stramineus* (Toyomasu et al., 1986); *P. ostreatus* and *P. columbines* (Toyomasu, 1989); *P. abalonus* and *P. florida* (Oiangtai et al., 1989), and *P. sajor-caju* and *P. florida* (Ming-Liang et al., 1989). Some

of the interspecific fusion products were capable of fruiting as such or after back crossing with one of the parent strains (Toyomasu, 1989).

Ramírez et al. (2011) obtained 11 hybrids by pairing *P. eryngii* and *L. edodes* neohaplonts. Additionally, 28 hybrids were obtained from di-mon mating by pairing different *Pleurotus* spp. dikaryons with *P. eryngii* neohaplonts. Kaur and Kapoor (2014) attempted protoplast electrofusion for development of somatic hybrids between *P. florida* and *P. sajor-caju* and produced hybrids yielding significantly better than the parents.

Djajanegara and Masduki (2010) carried out protoplast fusion between white and brown oyster mushrooms to obtain high productivity and long storage life. The regeneration of protoplasts is a time consuming and difficult process but has been performed successfully in species of *Pleurotus* and *Volvariella* (Reyes et al., 1998).

Mallick and Sikadar (2014) produced and characterized somatic hybrids *P. florida* and *L. edodes* through PEG-mediated protoplast fusion. Hybridity of the newly developed strains was established on the basis of colony morphology, mycelial growth, hyphal traits, fruiting body productivity, and ISSR marker profiling. Protoplast fusion between *P. ostreatus* and *P. djamor* was carried out by isolating protoplasts from 4-day-old monokaryotic mycelia cultured on malt extract broth.

Polyethylene glycol (PEG)-mediated protoplast fusion between *P. florida* and *Lentinus squarrosulus* successfully developed 12 *pfls* somatic hybrids using double selection screening method. Hybridity was proved by the colony morphology, mycelial growth, hyphal traits, fruiting body parameters and RFLP profiling of rRNA-ITS region (Mallick and Sikdar, 2015).

4.2.6.4 Di-Mon Mating

AHR Buller, a famous Canadian mycologist, demonstrated that homokaryotic mycelia in basidiomycetes could be dikaryotized by dikarons, that is, unclamped mycelia could be induced to form clamp connections (Buller, 1931). This phenomenon was referred as Buller phenomenon by Quintanilha (1937) and as di-mon mating by Papazian (1950). The results Huamin and Wei (1989) obtained on the basis of isoenzymes studies of mating of *P. sapidus* were different from the view that fusion of hyphae is followed by migration of two nuclei of dikaryons into monokaryons and reaches hyphae (Rawitscher, 1933) but identical with conclusions of Buller (1931). The results showed that it is reliable, simple, and time saving to use Buller phenomenon to carry out hybridization. Ingold (1984) has also reported the fusion of one, occasionally two, hyphae of *P. ostreatus* with its ungerminated basidiospore and has termed this as "homing."

4.2.6.5 Non-Conventional Breeding

Ramírez et al. (2000) advocated development of molecular tools for *P. ostreatus* breeding such as: (1) molecular markers genetically linked to mating factors in order to speed up the time required for selection of compatible mates, (2) a linkage map based on anonymous, functional, and genetic markers, (3) the molecular karyotype of *P. ostreatus*, and (4) molecular markers that allow the mapping of QTL of agronomic interest that can be selected for during the breeding process.

The conventional techniques in this species are much more effective than in *Agaricus bisporus* as all spores on germination give rise to monokaryotic hyphae, dikaryotization can be easily recognized by the presence of clamp connections. However, molecular techniques play a role in enhancing precision and also achieve better gains through assured identification of monokaryons and dikaryotization using DNA based technologies and such techniques can also help to identify markers linked with desirable traits and help in marker aided selection before performing actual fruiting trials. Gene transfer can take place through transformation, uptake

of foreign DNA, protoplast fusion, electroporation, and so on. Some of the non-conventional breeding methods are discussed below.

4.2.6.5.1 Genetic Transformation

As discussed earlier in the section on *Agaricus*, it is now possible to integrate foreign DNA into a cell, meaning that the genes from any organism can be transferred to another organism. The cells can be transformed by using restriction enzyme–mediated integration (REMI), electroporation, gene gun (particle bombardment), or *Agrobacterium tumefaciens*-mediated transformation.

REMI transformation uses restriction enzymes to increase transformation efficiency (Cheung, 2008). The term *electroporation* was coined by Neumann and Rosenheck (1972) to refer to the phenomenon of an ephemeral high-permeability state on the cell membrane due to pore formation following exposure to an electrical field. Gene gun (particle bombardment) is the introduction of DNA into intact cells or tissues by using high-velocity micro-projectiles via a mechanism that breaches cell walls and cell membranes. Heavy particles, such as gold, tungsten, and platinum, with adequate momentum to penetrate into the appropriate tissue are used as carriers. This method has been used in transforming *P. ostreatus* (Sunagawa and Magae, 2002) and *Volvariella volvacea* (Guo et al., 2005).

Agrobacterium tumefaciens-mediated transformation: The genetic improvement of a microorganism can also be achieved by a plasmid mediated transformation system. Bundock et al. (1995) and Chen et al. (2000) devised a highly efficient, convenient, and expeditious genetic transformation system for *Agaricus bisporus* based on the *Agrobacterium*-mediated fungal transformation method, originally described for *Saccharomyces cerevisiae*.

Using a DNA-mediated transformation technique, a molecular breeding approach to isolate *P. ostreatus* strains with enhanced productivity of its versatile peroxidase MnP2 was conducted by Tsukihara et al. (2006). A recombinant Mnp2 construct under the control of *P. ostreatus* sdi1 expression signals was introduced into the wild-type *P. ostreatus* strain by co-transformation with a carboxin-resistant marker plasmid. A total of 32 transformants containing the recombinant MnP2 sequence were isolated on screening with specific amplification by PCR. Productivity of MnP2 in the recombinants was evaluated by the decolorization ability of Poly R-478 on agar plates in the absence of Mn2+. Recombinant *P. ostreatus* strains with elevated MnP productivity were successfully isolated. One of the recombinants, TM2–10, was demonstrated to secrete recombinant MnP2 predominantly on a synthetic medium containing 15 mM ammonium oxalate, which was confirmed by reverse transcription PCR (RT-PCR) and isozyme profile analysis using anion-exchange chromatography. The benzo[a]pyrene-removing activity by fungal treatment was also analyzed using the isolated recombinant strains. Several publications exist concerning transformation of *P. ostreatus* and *P. florida* (Byun et al., 1989; Peng et al., 1992; 1993, Lemke, 1994; Herzog et al., 1995; Yanai et al., 1996).

4.2.7 Desired Traits

4.2.7.1 Yield

Yield and related traits like appearance, taste, texture, and quality are important parameters which are given due consideration. Strains that are able to give good performance on different substrates are always desirable. Work on genetic improvement has been going on for many years in a number of countries. For example, new strains of *Pleurotus* were developed at the Genetic Unit at the Mushroom Research Station at Bordeaux (INRA), which were marked by Somycel as 3001, 3014, and 3030 (Imbernon et al., 1983a,b). Les I strain of *P. ostreatus* was developed in Bulgaria (Kostadinov et al., 1987), which had higher protein content than wild strain (20.2% on dry matter basis vs 15.9%). Thereafter, a number of

commercial strains have been developed. The harvest of certain high yielding strains is strongly affected by the environment. Thus, strains and culture techniques have to be harmonized (Eger et al., 1979).

4.2.7.2 Sporelessness

Sporulation following meiosis is an essential process for sexual reproduction in mushrooms, but the release and spread of many spores from the developing fruiting body can cause several serious problems during commercial cultivation of mushrooms; one of the most serious problems is the inhalation of spores by workers, which can cause respiratory allergic reactions such as hypersensitivity pneumonitis and asthma (mushroom worker's lung). This disorder, first reported around the 1970s (Sakula, 1967; Jackson and Welch, 1970), is recognized medically as farmer's lung, which is an occupational health concern in the cultivation of various mushrooms (Matsui et al., 1992) and *P. ostreatus* (Jacq.) P. Kumm (Vereda et al., 2007). Oyster mushrooms normally produce large numbers of spores and these can be a cause of allergies to handlers. Traditionally mushrooms produce billions of spores floating in the air which not only cause health problems such as lung allergy and fever attacks but also lead to the blocking of climate installations and result in higher energy costs. Sonnenberg et al. (1996) estimated that 200–600 million spores are released per gram of tissue in 24 hours. As reported by Helbling et al. (1999) spores of *P. pulmonarius* cause specific IgE-mediated acute rhinoconjunctivitis and asthma in sensitized individuals. *P. ostreatus* spores cause exogenous allergic alveolitis (Senti et al., 2000). *Pleurotus* spores also cause hypersensitivity pneumonitis (Saikai et al., 2002). So, it is important to lower the spore load in the environment by developing low sporing strains or sporeless strains. Eger (1970) found and preserved a sporeless strain. Together with her coworkers she described new breeding possibilities, in particular for sporeless strains, by means of homokaryotization methods (Leal Lara and Eger-Hummel, 1982; Dickhardt, 1985). Chang et al. (1985) have also reported sporeless strains of *P. ostreatus* and Ohira (1979) reported sporeless strains in *Pleurotus*. Since 1984, commercial strains that are virtually sporeless have been developed. Somycel started marketing two sporeless *Pleurotus* strains; strain 3200 (oyster shaped brownish gray color) and strain 3210 (trumpet shape and gray color).

Advancements have been made in reducing spore load. Obatake et al. (2003) isolated a sporeless mutant dikaryon in *P. eryngii.* New dikaryons formed by crossing between one component monokaryon from mutant and 12 different wild-type monokaryons from three other wild-type dikaryons were all sporeless, whereas normal fruiting bodies were formed when crossing between other monokaryons and the same wild-type monokaryons. Pandey and Tewari (2004) developed a low-spore producing strain of *P. florida* by crossing sporeless *Pleurotus* mutant (dikaryon) with monokaryons obtained from sporulating parent, *Pleurotus* 572.

The large number of spores released by the cultivars also gives rise to other problems, such as damage to cultivation facilities, spread of mushroom disease (Grogan et al., 2003), reduced commercial value (due to spores deposited on the mushrooms), and depletion of genetic diversity in the natural population of the mushroom species that are cultivated (Hibbett and Donoghue, 1996; Obatake et al., 2003). The fruiting bodies of oyster mushrooms, including *P. ostreatus* and *P. pulmonarius* (Fr.) Quél, begin to release considerable numbers of spores at a very early stage in development and continue to do so throughout maturation. Eliminating overproduction of spores from the mushrooms is expected to yield many benefits, financial and otherwise, for mushroom cultivation.

Sporulation-deficient (sporeless) mutants are remarkably helpful for preventing these problems in mushroom cultivation. To date, spontaneous sporeless mutants have been found in *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys, and Moncalvo (Day, 1959; Gibbins and Lu,

1982), *Schizophyllum commune* Fr. (Bromberg and Schwalb, 1977), *L. edodes* (Hasebe et al., 1991), *P. ostreatus* (Eger et al., 1976), and *P. pulmonarius* (Ohira, 1979). Artificial mutagenesis using UV irradiation and chemical compounds has also been carried out to generate sporeless mutants of some mushroom species, including *C. cinerea* (Takemaru and Kamada, 1972; Kanda et al., 1989), *Agrocybe cylindracea* (DC.) Maire (Kiuchi, 1998; Murakami, 1998), *P. ostreatus* (Imbernon and Labarére, 1989; Pandey and Ravishankar, 2010), *P. eryngii* (DC.) Quél (Obatake et al., 2003), *P. pulmonarius* (Imbernon and Labarére, 1989), and *P. sajor-caju* (Berk and Br.) (Pandey and Ravishankar, 2010). Sporeless strains are commercially cultivated for only three species of mushroom: *A. cylindracea* (Murakami, 1993), *P. ostreatus* (Baars et al., 2000; Baars and Hesen, 2008), and *P. eryngii* (Obatake et al., 2006). Although sporeless strains are very valuable as breeding materials, traditional breeding for integration of the trait requires extensive time and labor because sporeless traits often link to significant adverse traits for commercial cultivation. The removal of these adverse traits has been the major obstacle in sporeless breeding. Thus, the isolation and characterization of the causal gene of the sporeless trait not linked to the unfavorable traits would be very beneficial and would lead to the establishment of versatile sporeless breeding using an approach employing targeting induced local lesions in genomes (TILLING) (McCallum, 2000) for various cultivated mushrooms in addition to *P. pulmonarius*.

Potential realization of the development of a sporeless fruiting body is especially promising in hybrids involving species with different chromosome numbers (Slézec, 1984). Measurements of relative content of DNA of cell nuclei do indicate the presence of different levels of ploidy with different chromosome numbers in various *Pleurotus* species (Toyomasu, 1989). The success obtained in isolation, fusion, and fruitification of interspecific fusion products will help to introduce new genetic diversity. Genetic engineering can also be of help in breeding new strains with enhanced lignocellulosic degrading ability (Ilu, 1989).

Okuda et al. (2013) used a *P. pulmonarius* (Fr.) Quél sporeless strain to identify and characterize the single recessive gene controlling the mutation. The 3853-bp *stpp1* gene encodes a protein of 854 amino acids and belongs to the MutS homolog (MSH) family associated with mismatch repair in DNA synthesis or recombination in meiosis. Gene expression analysis of the fruiting body showed that this gene is strongly expressed in the gills. Phenotypic analysis of disruptants formed by gene targeting suggested a reproducible sporeless phenotype. Mutants deficient in a functional copy of this gene have no unfavorable traits for sporeless cultivar breeding, so this gene will be an extremely useful target for efficient and versatile sporeless breeding in *P. pulmonarius* and various other cultivated mushrooms.

A new sporeless oyster mushroom has been developed by Plant Research International, Wageningen, the Netherlands by using molecular marker technology. After crossing various oyster mushroom cultivars, this analytical technique can be used to rapidly identify progeny with the highest chance of sporelessness (Okuda et al., 2009).

4.2.7.3 Wide Temperature Range

Temperature is one of the most important factors affecting mushroom development and introduction in new areas (Marino et al., 2003). In *Pleurotus* we have different species that can fruit in the temperature range of 15–30°C. Also, many of the species can be easily intermated. Mycelial growth variation in different *Pleurotus* species has been observed (Mehta and Bhandal, 1988).

From an adaptability point of view, it is desirable to have strains that can colonize substrate and fruit normally under a wide range of temperatures. Correlation of time to initiation of primordial and eventual yield has been reported (Wang and Anderson, 1972). Using this selection

criteria, it should be possible to screen for high yielding strains at different temperatures without going for complete yield evaluation.

In Hungary CV H-7, a cross between the P.5 winter strain of *P. florida* and the north American summer strain, can tolerate a temperature range of 8–25°C. Likewise, another good yielding strain producing quality mushrooms (CV G-24) was developed by crossing winter strain VL 6 with a summer strain. The temperature range of CV G-24 is 12–22°C. H7 is currently the most widely cultivated CV in Hungary followed by G-24 (Martonffy et al., 1987).

4.2.7.4 Chemical Tolerance

From the growers' point of view, successful mushroom varieties should be able to tolerate chemicals that can be present in waste products which are broadly used for substrate (Eger, 1978). Chemical sterilization methods have been developed for *Pleurotus* cultivation (Vijay and Sohi, 1987) and it may be desirable to develop strains highly tolerant to chemicals used in sterilization. The selection for chemical tolerance/resistance can be done easily at spore level (Eger, 1978).

4.2.7.5 Bioactive Molecules

Patra et al. (2011) developed a hybrid mushroom that produces an immunoactive polysaccharide by the protoplast fusion between *P. florida* and *V. volvacea.* Similarly, hybrid mushrooms producing anti-thrombin agents were developed by the protoplast fusion between *Laetiporus sulphureus* and *Hypsizygus marmoreus* (Okamura et al., 2000) in which *L. sulphureus* was the producer of anti-thrombin agents, but difficult to grow. Hence its limitation was overcome by generating fusant strains with *H. marmoreus* which has a well-established cultivation system. Breeding for lovastatin content, ergosterol and other nutraceutical parameters and identifying environmental conditions maximizing expression of genes controlling the production of such chemicals can help to enhance the nutraceutical value of mushrooms.

4.2.7.6 Other Traits

In some cases, the aim may be to alter only one of a few traits for which mutation breeding or gene transfer through recent biotechnological traits can be of use. One such problem is production of white sporophores in *P. sajor-caju*, the commonly grown species in our country. Ilu (1989) described a new strategy for breeding edible fungi through the enhancement of their lignocellulosic degrading ability by genetic engineering. There can also be commercial application if such traits are given due consideration.

4.2.7.3.7 Environmental Bioremediation

Plant cell wall lignin is the most abundant aromatic polymer on the earth and it is resistant to biological attack by most microorganisms. However, white rot Basidiomycetes degrade lignin and also recalcitrant environmental pollutants to carbon dioxide and water. *P. ostreatus* has the ability to degrade xenobiotic pollutants like pentachlorophenol, dioxin, and polycyclic aromatic hydrocarbons (PAHs) (Hirano et al., 2000; Kubatova et al., 2001). Wolter et al. (1997) investigated the degradation of eight unlabelled highly condensed PAHs by *P. florida.*

4.3 Conclusion

Although various approaches can be tried and exploited to solve specific problems, to begin with it may be desirable to go for inter-strain hybridization through conventional approaches. Our knowledge of the procedures is not a limiting factor anymore and with the support of molecular techniques it is possible to achieve targeted outputs. The availability of germplasm

is still a constraint. Despite the significance of breeding, the efforts are not at par with the needs. Germplasm collection and genetic improvement is a continuous process. With the extent of growth in the industry and demand for unique cultivars in all mushrooms, there will be an increase in breeding efforts. But germplasm collection cannot wait. We need much higher numbers of accessions in all the mushrooms than are available across the globe.

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5

Spawn Production

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This chapter deals with the production of spawn on a continuous basis, with a focus on today's advanced level techniques in the professional industry. The author is research manager at a Belgian spawn production plant called Mycelia,[1] which produces over 20 metric tons of spawn per week of over 150 strains and species. It is also a school for professional education on the production of spawn, of sterilized substrates and of lignicolous mushrooms,[2] a spawn lab consultant, and a research center on applied mycology.

The following pages contain hard-won information on a number of spawn production methods. It does not elaborate on the one-time production of spawn, which is characteristic for academic production or extremely small scale production[3] and where the rules can be less strict. In spawn production, continuity and scale of production are crucial: *the larger the production, the larger the risks of failure*. In other words: with every expansion step taken, the internal rules and regulations, quality control, and product flow have to be tightened. For example: whereas for a production of 1 ton per week, an autoclave with one door is still an option, it becomes nearly impossible when the production reaches over 5 tons per week. The risks become too great and the benefits gained with a double-door autoclave are too overwhelming to ignore.

This chapter is by no means complete. Producing spawn is a universe in itself, bound by the eternal complexities of life. After all, fungi are a miracle of life.

5.1 Our Spawn Industry Today

In today's industrialized world, it is economically viable to order spawn from halfway across the world and get it shipped to your doorstep. For example: a mother culture which was developed in Korea finds its way to the US, from where spawn is shipped to a mushroom substrate producer in Chile, whose harvest is flown into Brazil for sales on the fresh market. This may sound great, but it is as much a curse as a blessing. The curse is that price often has a negative effect

1 www.mycelia.be/en

2 Lignicolous mushrooms are wood-loving mushrooms, i.e., in natural environments, they primarily feed off lignin-containing food like wood and wood by-products. Examples are *Pleurotus ostreatus* – Oyster Mushroom, and *Lentinula edodes* – Shiitake Mushroom.

3 Specific for research settings, or for productions smaller than 5 metric tonnes per week.

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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on quality and on the survival of local initiatives. The blessing is that nowadays, one can successfully grow and eat mushrooms in almost every place on Earth.

The professional mushroom industry spreads like wildfire, but technology does not always follow the same pace. For example: there is a lot of information available on the Dutch production system of Agarics compost and Agarics mushrooms. However, not so much is accessible about the spawn that naturally goes with it. This much is sure: there are still a lot of places where local spawn is of inferior quality, because the producers often have no access to information, or because it is transported in abominable conditions. Some people advise substrate and mushroom producers to make their own spawn. In general, Mycelia does not advise this *unless* the spawn production facility is in a different place *and* is operated by different people. Over the years, there have been too many examples that went wrong. The golden rules for substrate producers are:

- If you can buy good and affordable spawn, don't produce it yourself.[4]
- If you can buy good local spawn, do not buy it from a distant producer.

In over 40 years, a large number of spawn companies have come and gone and many of them lost a lot of money. The reason is simple: the technology and investment needed for making good quality spawn is underestimated. Often, producers start off with a small lab and have good results. Then they start upscaling from there, but make only small investments in know-how and technology. Such an "upscaled" lab produces spawn of terrible quality with losses of 5–10% or more, which means nothing but trouble for substrate and compost producers. Consider the numbers: creating a spawn lab that produces 0.5 tons per week requires an investment of just €50,000 and simple production methods, but upscaling to 5 tons per week requires an investment of more than €1,000,000 and a lot of hi-tech, whereas a lab that produces 20 tons per week easily costs €3,000,000 and an even more complicated technology. Making spawn on an industrial scale is expensive and difficult.

Those people that start off on a *smaller scale*[5] are suggested to follow a training course before starting production, read a book, or search for information channels available on the internet. But be careful: there is quite a bit of false information around. Always be critical when consulting "specialists." Mycelia spends a lot of time mending badly designed projects.

Those people that start producing their own spawn on a *larger scale* will need to seek professional training on the production of spawn. Books and the internet will never be sufficient. Mushroom cultivation is not rocket science, it is a subtle art which takes a lot of feeling and years of experience.

5.2 Basics

5.2.1 Terminology

The following terms and definitions are used within this chapter:

Mother culture: also called culture, stock culture, primary culture, and vitro culture. It is the pure mycelium culture and its subcultures, usually grown on an agar medium in a petri dish or test tube.

Mother spawn: also called pre-culture spawn, spawn master and grain master. It is a pure mycelium culture transplanted and expanded from the stock culture.

4 Common exception to this rule: production of liquid spawn in large-scale bottle factories.

5 Spawn laboratories that have an output < 5 metric tonnes per week.

Spawn: also called mushroom spawn, spawn seed, mushroom seed, seed, and final spawn. It is a pure mycelium culture transplanted and expanded from mother spawn, and intended to inoculate a substrate or compost that will serve to grow mushrooms.
Substrate: the medium on which a mycelial culture grows.
Mushroom substrate: the substrate which is not used for further multiplication, but instead for the production of mushrooms.
Leap-off time: the delay with which a mycelium starts growing from the inoculum into the surrounding substrate. This time interval is also called the *lag phase* or *lag time*. Lag time is also the delay at which the substrate heats up compared to the sterilization chamber.
Hypha: branching thread of a fungus built from elongated fungal cells.

5.2.2 The Mushroom Production Pyramid

Spawn makers are part of a cycle. Spawn inoculates substrate, which eventually yields mushrooms. These are sold off to consumers, so finally, they have the final word. And everybody knows how difficult it is to please consumers: they want perfect sized and shaped mushrooms, every day. To achieve this, every step of the production process needs to be standardized.

This is a realistic example:

1 mother culture → 10 petri dishes → 50 l of mother spawn A → 1000 l of mother spawn B → 60,000 l = 42,000 kg of final spawn. At this point, the spawn is sent to a substrate producer in a refrigerated truck. If this producer spawns at 1%, he or she produces up to 4,200,000 kg of substrate and if the harvest is 30% of the fresh weight = an output of *1,260,000 kg of mushrooms*.

This resembles a pyramid with a very broad base (see Figure 5.1). If something goes wrong at the top of this pyramid, the effects bottom-down become ever more disastrous. Hence it is forever the responsibility of a spawn producer to maintain and improve the quality level of their products. It is the only way to win and keep clients. The pyramid also explains why substrate producers rarely produce their own spawn: every failure is paid in full and the financial advantage of producing one's own spawn hardly ever weighs up to the disadvantage of a large loss of revenue.

What makes spawn production so complicated?

- all competitors are invisible. Many grow faster than the mushrooms we are trying to grow and they are present in every environment, in astonishing quantities

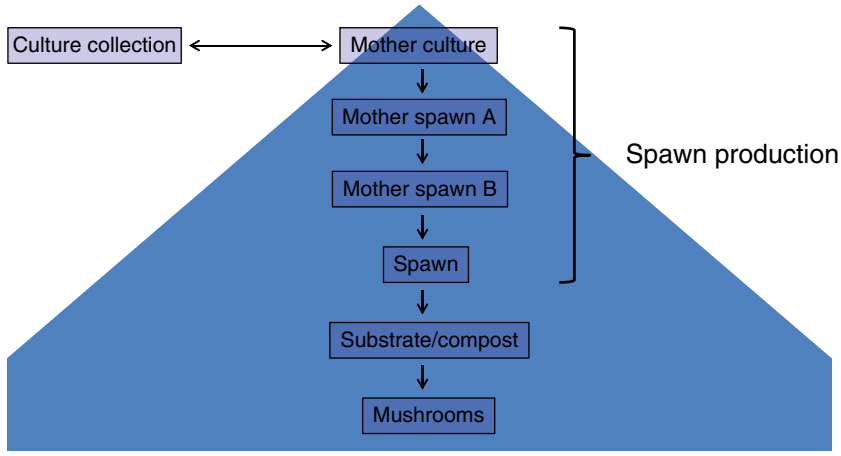

Figure 5.1 The mushroom production pyramid.

- spawn production is a process of multiplication, problems increase exponentially
- the necessary investment in infrastructure and know-how is considerable
- the revenues are small and the risks are high
- fungi are very sensitive to stress and degeneration.

5.3 Spawn Production Techniques

Interestingly, there is more than one good technique to produce good spawn at a professional level and there is no "best method" for unifying the industrial producers. On the contrary, there is a multitude of techniques and each one has both advantages and disadvantages. A simple geographical analysis reveals much about today's distribution of spawn production methods.

The most used method today is (1) *grain/sawdust spawn grown in small bags*. It is par excellence the method used in most Asian countries, including China, Japan, and India. But other techniques are catching up in this region, mainly liquid spawn inoculation (used in bottle culture) and grain spawn in larger bags. Small grain spawn bags are used in many other regions in the world too, like Africa and South America. This is the only solution if there is no opportunity to produce large grain spawn bags and/or where there is little opportunity to import such a product from abroad.

Two other systems are in a neck and neck race. These are (2) *Liquid spawn in fermenters*: this is mostly used in the production of bottle cultures in Asia and (3) *Grain spawn in large bags*: these are common in the European and North American countries, among others. They are either bulk sterilized or pre-bagged and then sterilized. Crossovers have also been known. (4) *Liquefied spawn* is created by some producers in the professional industry to inoculate one's own spawn. It is an important but highly specific technique. (5) *Grain spawn in bottles or jars* is not used as much due to practical concerns. However, bottles and jars are widely used for the production of mother spawn or in remote laboratories that have little chance of buying bags. (6) The use of *spore syringes* is the most obsolete of the available techniques. It has its merits, but not in the commercial and continued production of edible or medicinal mushroom spawn. For that reason, it will not be reviewed in this chapter.

The choice of production technique by a particular spawn laboratory is dictated by many factors, of which the most important are: (1) availability of spawn bags, (2) local climate, (3) economic constraints, (4) knowledge level, (5) conservation and transport, and (6) personal preference.

Spawn is in general sold per units of volume, mostly expressed in liters. This is due to a variability in the density of raw materials. Most bag filling machines operate without weight scales, but with a volume dosing head.

5.3.1 Grain/Sawdust Spawn Production in Small Bags

Most spawn producers, especially the multitude of smaller labs scattered around the globe, use small bags of 500 g to 1 kg for making grain spawn. Most of these are labs with a limited output, but quite a few manage to produce large quantities of 20 tons per week and more. Still, it is by far the most used technique by small labs, and today there are still a great number of those in the world. Small bags have certain advantages: they don't require embedded filters (although some do have filters inbuilt), so they are often used in combination with cotton wool stoppers or other top filters. Because of their small size, overheating and asphyxiation are rarely an issue. Also, it is an accessible technique in the sense that it can work in situations where there is almost no technology available. It is a good solution for still air boxes, inoculation

cabinets, or glove boxes with little or no air filtration. It has disadvantages too: it is a very labor- and energy-intensive way of working, and the losses are often considerable (5–25%). Exceptions do exist. Because of this generally lower quality, it is harder to build a long-term relationship with clients. Before the revolution in breathing bags in the 1990s, small spawn bags or other containers were almost the only available technique and everyone used it. Since then, many producers have been changing to larger bags.

Especially in Asia, sawdust spawn in small bags or bottles is essential for the automated bottle inoculation devices, and is also used for the cultivation of Shiitake on logs. In other regions, sawdust spawn is rarely used.

Technique: there is no general technique, since every lab develops its own system. An example of an inoculation technique of this type of bags is depicted in Figure 5.2. Most of the labs pre-boil small batches of grain until the kernels are soft (see Section 5.6.3). Examples of grain types are wheat, millet, sorghum, rice, and others. These are pre-bagged or pre-bottled

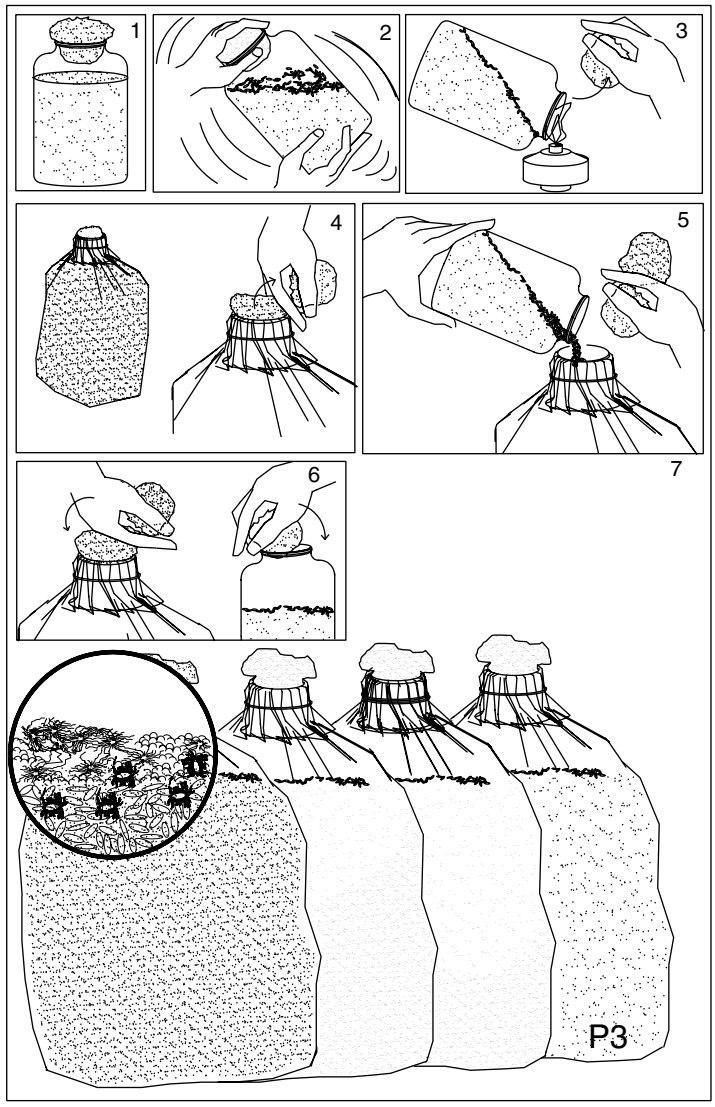

Figure 5.2 Example of inoculation practice of small grain bags without inbuilt filter.

1) Take the pre-culture spawn from the pre-culture refrigerator and disinfect it.
2) Mix the pre-culture well in front of the LAF.
3) Remove the cotton plug without passing between the LAF and the bottle. Flame the edges of the bottle.
4) Remove stopper from spawn bag.
5) Inoculate in front of the LAF. Do not move your hands between LAF and bag. In front of a large LAF, inoculate a series of bags at once.
6) Insert the stopper into the spawn bag. Do not re-use pre-culture bags!
7) Incubate the spawn bags until fully mature.

manually, the stopper is inserted and covered with a heat-resistant foil. After autoclaving, the bottles are inoculated one by one, in glove boxes or still air boxes. These boxes are disinfected inside, mostly by burning a chemical, for example chlorine tablets. The process often includes the use of an inoculation tool (not displayed here). After inoculation, the bottles are placed in an incubation room until ready.

5.3.2 Grain Spawn in Large Bags

This technique has been gaining momentum since the revolution in breathing bag technology (see Figure 5.3). A volume of 10 l of spawn behaves very differently to a volume of 5 l, which in its turn is incomparable to 1 or 2 l. The larger the volume, the more complex the gas exchange and heat balance, hence the need for a more complex spawn bag. Mycelial mass, strain, climate, and distance to filter are crucial factors. For some species, such as *Agaricus bisporus* and *Pleurotus ostreatus*, spawn bags of 10 or even 15 l work well, but for most species, volumes of no more than 5 l are preferable. Hence, bags must not only be adapted to the production system, but also to the volume and species they are designed to hold. The bags with filters crossing the complete bag surface display the most balanced gas exchange, so strips are, by definition, better than patches (Van Nuffel et al., 2016). A well-designed spawn bag earns itself back many times over.

There are two distinct ways of producing spawn in large bags, typically 5–10 l: bagging first and then sterilization and inoculation in individual bags *or* sterilization and inoculation in bulk and bagging afterwards. The difference in product flow is represented schematically in Figure 5.4.

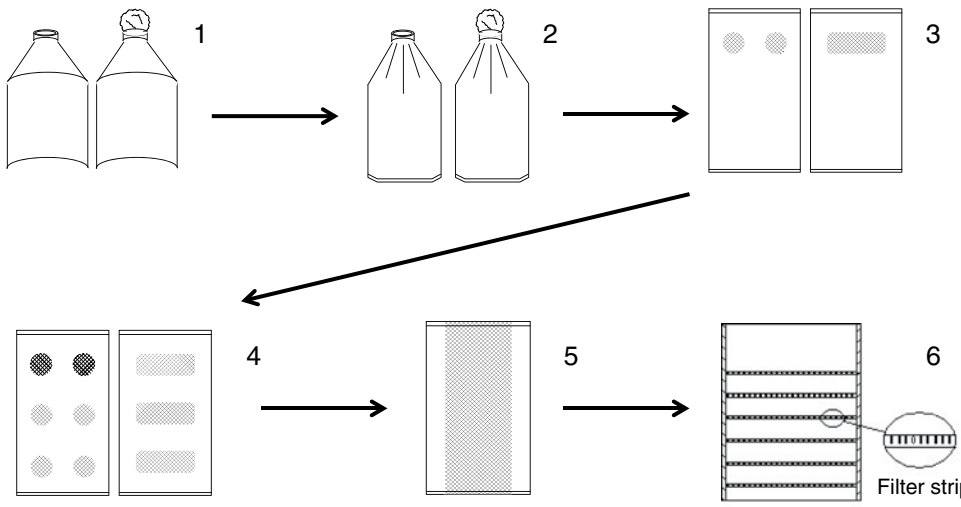

Figure 5.3 Evolution in breathing bag technology.

1) Bottles with stopper
2) Bags with stopper
3) Bags with filter patch
4) Bags with multiple filter patches
5) Bags with filter strip
6) Bags with multiple filter strips

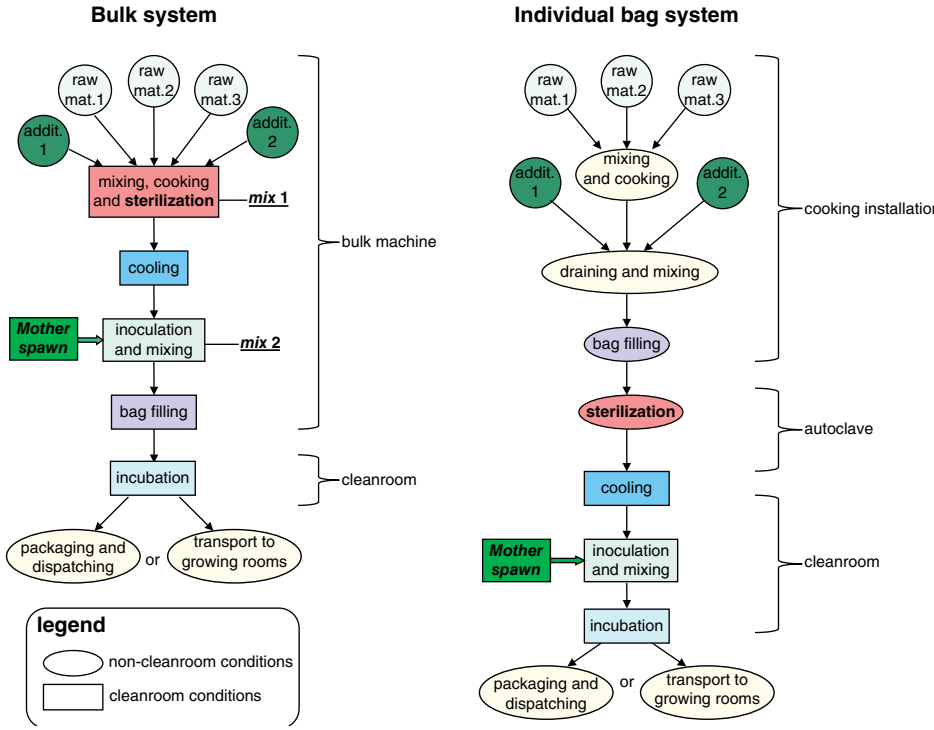

Figure 5.4 Comparison of product flow of two spawn production methods: sterilization in bulk or individual bags.

5.3.2.1 Individual Bags

This technique is comparable to the production of grain spawn in small bags (see above) in terms of product flow, with the exception that large bags are often re-shaken and small bags are not.

There are quite a few methods to make this system work, but the standard substrate preparation is basically as follow: first, the grains are boiled in water until the starch has gelatinized and the grains are softened but not opened. Then they are drained, and in most cases dried out until cool. During this phase, chalk and gypsum are added and the whole is mixed until homogenous. This needs to be gentle mixing, as little as possible so as not to damage the kernels. Damaged kernels guarantee a sticky substrate. Finally, the mix is bagged. Be aware that the bag type dictates much of the following steps.

Most bags have the filters facing up - not touching the substrate – after filling. Like this, they are placed on the autoclave trolleys, with the flaps unsealed. In this case, a "vacuum cycle" is the quickest and most efficient sterilization system. It only takes 2h 30 min, as compared to a standard "gravity cycle" of 5h (see Section 5.8.1). After autoclaving, the trolleys are cooled under sterile overpressure. When the centers of the bags have reached a temperature of below 30°C, they can be taken to the adjacent inoculation room, also under sterile overpressure. There, they are inoculated with mother spawn and sealed with the filters up, all in front of a laminar air flow unit (see Figure 5.5). The inoculation can be manual or automated, as desired. Then, the bags are shaken in order to distribute the inoculum. To obtain an even distribution of the inoculum, the headspaces of the bags need to be full of air, at least the same volume of the grain. Also, the shaking must be as complete as possible. A bag tumbler is a great help (see Figure 5.6). After

Figure 5.5 Manual inoculation of large grain spawn bags in front of laminar flow. Note the anemometer measuring the horizontal LAF's air velocity at the top and the sealing machine at the right.

Figure 5.6 Bag tumbler.

shaking, the bags are turned upside down, with the filters down, touching the substrate, and are placed on an incubation trolley. If the bags are not re-shaken, the bags can be put directly into their final shape. Important: this system only works with filters crossing the complete surface of the bag. Spawn bags with a patch are not turned upside down at any point. The disadvantages

of the latter are that (1) the gas exchange is much more irregular and (2) the bags take up much more space during transport (Van Nuffel et al., 2016).

A second option is to use bags that are filled and placed on autoclave trolleys with the filters facing down. They are autoclaved, cooled, inoculated, and sealed with the filters down, shaken, and put on incubation trolleys with the filters down again. The main risk is getting wet filters during autoclaving, which involves an extra risk of infections.[6]

Regardless of the production system, the trolley is then wheeled into the incubation room and incubated until ready. Many producers choose to re-shake the bags when the inoculation points have formed colonies of 3–5 cm diameter. The trolley is then placed into the final incubation room for another few days, till the mycelium has fully overgrown the substrate. The bags should contain a lot of air in the head space before re-shaking, so they must be positioned correctly after inoculation. It is also possible to add a larger amount of inoculum so as to avoid the re-shaking step. In this case, grain mother spawn, liquefied mother spawn, as well as liquid inoculum can be used. One needs to say that liquefied inoculum usually gives better results than liquid inoculum due to the shorter lag phase. A good and constant shaking protocol, right after sealing, is essential. Because this shaking is so vigorous and because the bags are big, the sealing machine must be top-notch. An interrupted or continuous heat sealer, or an ultrasonic sealer is excellent. But be aware that ultrasonic sealers are too expensive for this job.

Most spawn producers still underestimate the importance of the growing vessel. In mycelium production, a good breathing bag is top priority. The quality difference between a spawn in a patch bag and a filter strip bag is huge due to gas concentration gradients inside the bags.

When sterilizing bags in an autoclave, these need to be autoclavable, strong, transparent, sealable, and flexible. The filters also need to have the correct gas exchange capacity. A PP-bag with the filters crossing the complete surface of the grain volume is the best option available. Dealing with the bags, however, is not simple. A good spawn bag filter is hydrophobic, meaning it does not absorb water under atmospheric conditions. If the filters touch the grains during autoclaving, however, they will get saturated with nutrient-charged water. After cooling, this wet and nutrient-rich filter becomes the perfect breeding ground for weed molds. Their spores will land on the wet filter, sporulate, and feed off this micro-environment. Their hyphae can grow toward the inside of the bag and sporulate on the inside. On the other hand, infections that are already present in the bag can grow through the filter and sporulate on the exterior of the bag. Both these problems are common phenomena in "dirty" cold stores, where spawn is stored together with substrates or mushrooms.

Note: sawdust substrate is rarely produced in large bags.

5.3.2.2 Bulk

The bulk production of grain spawn is entirely different. The idea behind a bulk production unit is to combine as many steps in one machine as possible. This serves two causes: (1) to have fewer employees and (2) to upscale without adding to the inoculation risks. There are a number of different designs, but most work as follows: the ingredients are put into a pressure vessel which has an internal mixer (see Figure 5.7). This vessel is then heated up using either double-mantle heating or direct steam injection. Mixing, cooking, sterilization, and cooling are combined in one process. The used sterilization method is a simple gravity cycle with fast heating and cooling. The cooling step is the most critical: large batches are hard to cool down, and the

6 Another important technique is sterilization in bags without filter and after cooling, tipping over the grains into a second, sterile bag with filter strips, followed by inoculation, sealing and shaking. The first bag is discarded.

Figure 5.7 Example of a bulk vessel for the production of *Agaricus* grain spawn, inoculated with liquid mother spawn.

smallest leak in the system will cause contamination. After the content has reached 30°C, the mother spawn is added to the vessel and evenly mixed into the substrate, both in sterile conditions. This mother spawn is either liquid, liquefied, or grain-based. After mixing, the complete vessel is emptied into pre-sterilized bags, mostly polyethylene plastic. These bags are either provided with filter strips crossing the complete length of the bag, or with evenly distributed filters. Bags are not re-shaken, but are directly placed onto the incubation trolleys. Needless to say, the technical parameters of this production system are way more complicated than working with individual bags. If the technical side works flawlessly, it is a very balanced technique with limited risk. Moreover, the mixing of the inoculum into the sterile mass is nearly perfect, so the end product is standardized. But often, technical issues crop up, causing entire batches to be lost. This is the main risk of any bulk system: considering the size of the batches, the size of the losses can be equally large (e.g., if there is a problem with one mother spawn batch). The bulk production system has been developed in Europe and the USA and has been gaining momentum ever since its introduction. It has been the driving force behind the globalization of spawn sales. Nowadays, complete tumbling bulk production units can be purchased (see Figure 5.6), including liquid spawn fermenters for grain inoculation and sterile air systems. However, placing a bulk system in a room under sterile overpressure is still advisable.

Something to keep in mind is that bulk systems have been developed for the production of larger batches of a simple recipe. For small batches, tests, or complex recipes, individual bagging in an autoclave is more suited.

Note: There is also a crossover technique between bulk and individual, in which sterilization takes place in individual bags. After the cooling, the bags are emptied into a bulk vessel where inoculation and mixing takes place, after which the bulk volume is bagged again, this time in sterile bags.

5.3.3 Liquid Spawn

Liquid spawn can be used for a wide range of applications. The advantages are clear: it is a liquid, so it is easy to handle, easy to disperse, and easy to store. It is also available as a fully automated system with large or small fermenters, with little work, and a very fast increase in biomass. Mostly, it is not produced by spawn companies, but directly by producers of mushroom substrate. These companies control the whole production chain, from culture over spawn to mushroom substrate, often even including mushroom growing. For these companies, spawn is a cheap product. But because of the long lag phase (see later), it works best in facilities that have a very high level of hygiene. As a result, it is mostly used in very large bag and bottle farms in Asia where only very few species are cultivated on an equally large scale. Examples are the King Oyster and Enoki bottle farms in China and South Korea.

Liquid spawn can be pumped from one place to another, through tubing, so the inoculation process can be 100% automated. That has the advantage that people are much less involved, so there is much less chance of infections, but it has the disadvantage that the success depends on machines and on how well these machines are being maintained and cleaned.

Liquid spawn has a long lag phase: as explained in Figure 5.8, it takes a long time before the inoculation points inside an inoculated mushroom substrate start growing, often more than 3 days. This is due to the lack of a number of key enzymes like cellulose and laccase, which are present in grain spawn and even more in sawdust spawn. It takes the mycelium several days to catch up to this delay. A long lag phase is a big disadvantage for production systems where the leap-off speed of the spawn is of crucial importance, for example where the substrate is pasteurized or weakly sterilized. It has been shown, though, that the initial slow take-off is compensated by the later growth speed and that the final incubation time is the same as for grain spawn. This makes liquid spawn useful for mushroom substrates that have reached a very high degree of sterility. Most producers of sterilized mushroom substrates, however, do not attain sufficient levels of sterility, as this requires a high level of understanding in sterilization cycles and hygiene and there is a high initial investment in machinery. Also, because of the technicity of handling the liquid spawn on an industrial level, it is mainly usable in situations where there are very large batches of the same strain in small portions and where the size of the

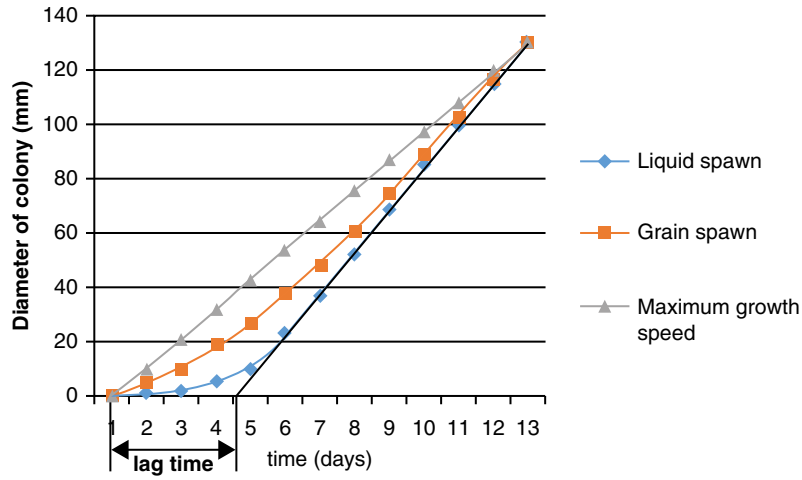

Figure 5.8 Lag time of liquid spawn vs. grain spawn of *Pleurotus eryngii*. If the amount of liquid inoculum is higher, the lag times are noticeably shorter (Source: Gruwez, 2003, http://bmcmicrobiol.biomedcentral.com/articles/10.1186/s12866-015-0592-4. Used under CC BY 3.0 https://creativecommons.org/licenses/by/4.0/).

production plant is equally large. It certainly has all the qualities required for inoculation in large small-bag and bottle farms. On the other hand, it is less suitable for smaller farms or in farms where sterility cannot be 100% guaranteed. Within spawn production sites, it can be used as mother spawn, to inoculate the final grain spawn.

An important factor when using liquid spawn is the degree of mixing. Liquid spawn has the major advantage that it provides the substrate with a huge number of inoculation points, but they start off with a certain delay. To gain the lost time due to the long lag phase, the liquid must be well dispersed throughout the substrate. This can be done either by intensive mixing, or by using some kind of spraying nozzle for inoculation. In large bottle farms, the liquid is sprayed into the bottle or injected with needles, but as the level of cleanliness is often top-notch, the effect of a shorter lag phase is very limited. Such large farms have exceptionally high levels of hygiene.

Technique: liquid spawn starts with either (1) agar medium or (2) spore mass inoculation.

1) Inoculation from a single-strain agar plate
   A petri dish or slant with a culture is used to inoculate a glass container, for example a 300 ml Erlenmeyer. The vessel is filled with 200 ml of medium. The neck is shut off with a suitable filter, such as a cotton wool stopper or a membrane filter. After sterilization and cooling in a sterile environment, the bottle is inoculated with a small piece of agar. After 2–4 days of fermentation at the proper temperature (depending on the strain), a second stage of expansion can occur into a larger volume of sterilized broth, resulting in yet another 25- to 200-fold increase of the volume of the original mycelial mass. Like this, the liquid spawn is expanded until it is ready to be used in mushroom substrate. In each step, an inoculation volume of 2.5–10% must be taken into consideration (Kirchoff and Lelley, 1991). A heavier inoculation rate always results in faster growth. The final incubation takes place in fermenters, these are large stainless steel vessels that are filled with a medium. The vessels must be continuously vented with sterile air to exhaust volatile metabolites and to supply fresh oxygen. Most of them have some kind of stirring system to break up the mycelial mats and clumps as they form through the liquid (Itävaara, 1991). The latter must be broken continuously so as to prevent clogging of the fermenters and tubing. If the fermentation is allowed to go too far, the liquid spawn becomes too dense and cannot be used anymore for inoculation. Cleaning the fermenters and inoculation system can be a dreadful job at that point (Geers, 2002; Emmerechts, 2003; Gruwez, 2004).
2) Inoculation from a spore mass
   A sterilized mushroom substrate, for example, woodchips or sawdust, is inoculated with a suitable strain and incubated in aseptic conditions. Then, mushrooms are grown and harvested from this substrate, again in perfectly aseptic conditions. These mushrooms are then transferred into the starter bottle – in this example, a 300 ml Erlenmeyer containing 200 ml of liquid. Soon, millions of spores are washed into the broth, germinating all at once, and producing a complex multispore culture with countless individuals (Scrase R, 1995). After the initial inoculation, the technique is identical to previously.[7]

There are also producers that use a crossover technique between liquid spawn and grain spawn. In this case the mother spawn is produced in liquid form and is then used to inoculate the second generation of mother spawn on a grain recipe. In other production sites, liquid spawn is multiplied to inoculate the final spawn. Liquid spawn in this form, of course, can be used with success in spawn laboratories of any size.

7 When using spore mass inoculation, countless individuals interact in the same medium, thus producing a true multispore culture. At the time of writing, the author has no knowledge of research indicating that this technique has striking advantages or disadvantages over liquid inoculation with agar medium of one single strain.

5.3.4 Liquefied Spawn

Simply put, liquefied spawn is solid spawn made liquid by adding sterile water followed by aseptic mixing.[8] The end product is a sterile slurry. Liquefied spawn can be pumped from one place to the next, making it also very practical for large-scale inoculation of bulk systems (see Section 5.3.2.2) or inoculation of grain spawn in large bags. It has most advantages of liquid spawn, but with the added advantage of a shorter lag phase. In order to prevent the medium separating into liquid and solid phases it must be continuously mixed. Just as for liquid spawn, the machines used for automated inoculation must be kept meticulously clean. Since the particles are larger, the pipework and pumps are more susceptible to blocking than when using liquid spawn, but the inverse is true for the mixing vessels.

Liquefied spawn has a shorter lag phase than that of liquid spawn. Key enzymes like cellulase and laccase are present in the solution.

Just like liquid spawn, liquefied spawn has an important advantage over grain-based inoculum: if mixed well, it creates a very high amount of inoculation points in the target substrate. Each of these points grows out to be a colony and as a consequence, the end product is very homogenous. To obtain a comparable homogeneity with grain inoculum is not simple. But liquefied spawn cannot be kept for a long time, unless the water is not yet added.

The substrate of liquefied spawn can be any grain spawn in mixed form, but a powdery substrate is even more appropriate, like flour or a combination of flour with mixed perlite.

5.3.5 Grain Spawn in Plastic Bottles or Glass Jars

The production of spawn in glass jars and plastic bottles is comparable to that of grain spawn in small bags, more precisely to those which are provided with one single filter patch at the top, or a stopper with rubber band or equivalent. The use of glass jars is very laborious; for that reason nobody except for a few smaller laboratories uses such receptacles for the production of commercial spawn. But they are used all around the world for the production of mother spawn, more for practical reasons than for anything else (Stamets, 2000).

In some developing countries on the other hand, obtaining proper bags is very difficult. In these laboratories, glass jars or liquor bottles are the only option.

Note: using glass or plastic bottles might be viewed as an environmentally friendly way of producing spawn, but often these bottles are smashed and thrown away after use.

5.4 Strain Selection

Strain selection is a very complicated issue which takes years of practice and experiments. Since it is such a complex science, it is discussed in Chapter 4. In industrial terminology, strain selection is a vague term referring to a number of practices: isolation, cloning, selective breeding, and genetic engineering.

Isolation is the process where a single genotype is taken from its habitat and then grown without contaminants. The resultant axenic culture is called an "isolate." The basic techniques for such isolation are (1) cloning from a mushroom and (2) isolation of mycelium of a specific strain from a sterilized medium (spawn, petri dish, other). These two techniques are discussed further in sections 5.5.3 and 5.5.4. Another, more obsolete isolation technique is (3) selecting

8 It is also obtained by adding sterile water to a solid medium, shaking and then extracting the water from the medium. The water then contains floating mycelia, enzymes and other beneficial elements.

from a spore mass,[9] where a pure mycelium culture is grown from a large quantity of spores. It is rarely used as the results are highly unpredictable. Most novel strain research now focuses on the (4) selection of hybrids and on (5) genetic engineering.

Genetic engineering is a very complicated topic and will not be further discussed. To the knowledge of the author, genetic engineering is not common in the creation of new strains in the spawn industry.

5.5 Strain Preservation and Degeneration

Spawn producers work with strains which have a number of outstanding qualities such as high crop yield, perfect mushroom size and shape, long shelf life, mycelial adaptation to climate, short incubation and ripening time, and so on. These strains are typically (1) isolated from nature, (2) selected by hybridization, (3) genetically engineered, (4) bought or (5) copied from another company. Most spawn companies copy each other's strains.[10] Once a strain is selected or genetically altered into a desirable offspring, this particular individual is asexually multiplied, that is, without altering the primary qualities of the strain itself. The idea is to do this indefinitely. The trouble is: nature did not design life to continue forever. A fungus – like all living organisms – is born, lives for a while, and eventually dies. Humans can prolong the life of a particular fungus by duplicating and multiplying it, but this cannot be done indefinitely. As life moves on and the biological age of the individual increases, genetic and other defects crop up and finally lead to so-called "degenerations." They are probably the spawn producer's worst nightmare.

What is degeneration? In the mushroom business, it is a very vague term used for any genetic alteration causing – mostly phenotypical – aberrations. In other words: the harvest fails. The mushrooms can have hard gills, long stems, or different colors, there may be less mushrooms or in some cases no mushrooms at all. The list is virtually endless. Some of the degenerations seem to be associated with stress, such as rapid thawing, drought, or asphyxiation. Others appear to be genetic mutations and some are connected to biological aging (senescence) when they are grown in spatially or nutritionally limited habitats. It is not easy to research this topic; the rules that apply to other organisms such as "birth, growth, sustenance, death" do not apply to the filamentous fungi as they do to, for example, plants and animals.

The topic of degeneration is still largely unclear and is being researched. Research done so far has not delivered a method to trace instability on a mycelium level (mother cultures) nor a method to maintain strains in such a way that degeneration is prevented (Raamsdonk et al., 2001). Spawn companies and labs are just using their experience to maintain strains to their best knowledge. Pre-testing new lines on a limited scale can sometimes prevent disasters on a large scale, but it cannot prevent occasional occurrence of degenerations. Nothing is known of the fundamental processes or triggers that cause degeneration (Horgen et al., 1996).

An important question is: how often do degenerations happen in mycelium cultivation? The answer is: rather often. If a mycelium culture is continuously multiplied, it won't be long before some kind of genetic defect crops up. An important rule is: *keep the number of multiplications*

9 If you take a soil sample for example, it will contain up to 10 spores per gram dry weight. The sample will commonly include more than 20 different species of fungi, both spores and hyphae. To isolate a single fungus, you need to use a method to reduce this density of propagules such as soil dilution, soil plating or hyphal extraction (Warcup, 1955).

10 An interesting fact: it is suspected that almost all cultivated white button mushrooms in the world are copies or (in)direct descendants of only three individuals (Dutch research, source not revealed).

limited to avoid degenerations. In fact, ANY bag of spawn sold by professional companies should be only a few multiplications away from the original individual. This obviously reduces the chances for degeneration to an acceptable degree. Spawn producers should realize the immense risk of degeneration they are running should they not refresh the original strain regularly.

There are three main techniques to keep the amount of multiplications limited: conservation, subculturing, and strain refreshment. There are a number of conservation techniques, such as deep freezing, cooling, keeping in a watery solution in a fridge, submersion in paraffin oil, and others. We will now discuss the most common ones.

5.5.1 Deep Freezing

Mycelium can be kept virtually indefinitely when it is frozen at extremely low temperatures. Recovered cultures from deep-frozen containers have the advantage that they contain the genetic source code, frozen in time. It is unknown to science for how long mycelium can survive in such frozen conditions, but there have been experiments extending well over 25 years which still produced active mycelium, even after repeated freezing and thawing (Kitamoto et al., 2002). Note, however, that some recovered strains show signs of degeneration, possibly due to water stress during freezing (Horgen et al., 1996).

Deep freezing requires either liquid nitrogen at −196°C, or a cascade-type electric freezer which can achieve −80°C or less (see Figure 5.9), the latter being more practical to use. It is a well-documented technique: first, a strain is inoculated directly from an agar plate on a suitable, stable carrier with the same technique of regular mother culture inoculation. This must of course be a reliable strain, of which crop results are known and documented. Lignicolous mushrooms are grown onto a lignin-rich carrier (e.g., a small piece of wood), compost mushrooms on a compost-rich carrier (e.g., a piece of composted straw). Once entirely incubated, the carrier is put into a cryovial, which is then filled with a freezing solution. After a few hours of acclimatization, the cryovial is put into the freezing device. Contrarily to some plant and animal cells, most Basidiomycete cells survive the quick transition without being damaged. A number of solutions are suitable, of which a 10% glycerol solution is the most commonly used. One such cryovial can be thawed when needed, typically slowly so as to soften the thermal shock. Most Basidiomycetes and fungal spores recover within 2–6 days after thawing, even after quick freezing and thawing (Dahmen et al., 1983; Kitamoto et al., 2002).

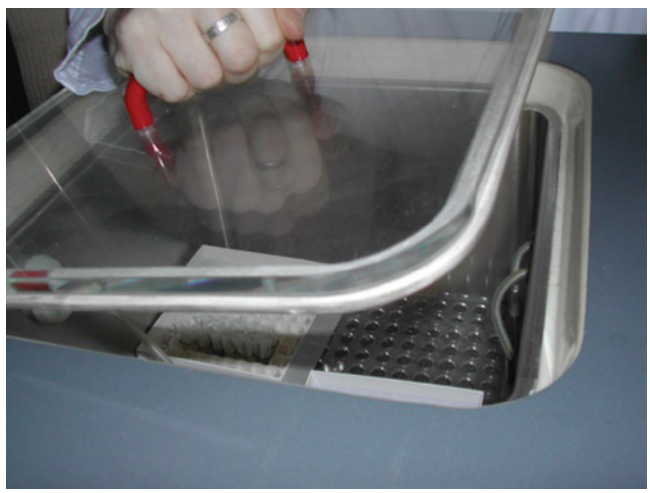

Figure 5.9 Keeping cryovials in a deep freezer.

Notes:

- Not every spawn producer should have a deep freezer, as there is always the possibility these days to buy back an original strain to avoid degeneration. Also, a company which chooses to embark on the deep-freezing path should realize the risk of mechanical breakdown of these machines and take appropriate measures.
- Make a habit of testing the quality of the carrier by incubating some material on a test plate (a rich medium petri dish like MEA with sugar) while you are preparing the cryovials.

5.5.2 Cooling

The simplest and least time-consuming technique for strain maintenance is cooling. Simply placing a living mycelium product in the cooler slows down its metabolism, prolonging its life as if during a long, cold winter (see Figure 5.10). The ideal storage temperature for most cultivable species is 1–3°C, except for several temperate and subtropical species such as the *Pleurotus djamor* syn. *P. salmeostramineus* and *Volvariella volvaceae*. These are typically stored at around 10°C. This way, most species can be kept in good condition for 6–12 months in a petri dish and over 3 years in a test tube. Eventually, consumption of available nutrients, toxification of the environment, increase in biomass, and desiccation cause the fungus to die. There is no set time for how long a culture can be kept, it depends largely on the storage quality and on the culture's purpose. If it is meant for multiplication it should be as young as possible, but if it is meant merely as a strain backup, it can be kept for years on end. Slants or petri dishes older than 6 months usually serve as backups only.

Figure 5.10 Keeping cultures in a culture collection.

Note:

- Watch out! Molds grow well where there are thermal bridges, such as refrigerator doors. And because refrigerators cannot be kept under sterile overpressure, each item should be individually packed inside a breathing bag to avoid contamination and cross-contamination (see Figure 5.11). Some refrigerator doors "fall" into place upon closing, that eliminates most of this problem. A good door joint and regular cleaning take care of the rest.

5.5.3 Subculturing

As stated previously, fungi cannot be indefinitely held in a refrigerator as a mother culture. Once in a while, they are refreshed through the subculture method. For that purpose, one piece of the parent culture is transferred onto a fresh carrier and incubated till fully grown. Subsequently, it is placed in the refrigerator as a replacement of the old carrier. Spawn companies often combine this replacement with the creation of lines – also see Section 6.1.

When a strain is considered suitable for multiplying into mother spawn, one mother culture slant is multiplied into lines. The basic idea behind creating lines is: *spreading the risk of production failure over a number of parallel offspring*. In other words: if something goes wrong with one line, it is easy to discard it without endangering the complete production. For small productions – under ½ ton per week – two or three lines are sufficient. From there onwards, the amount of lines goes up gradually.

Technique: see Section 5.6.2.

Note: When subculturing, the new culture is, by definition, one generation away from the parent culture. If this is your only strain preservation method, it is advisable to keep track of the number of generations.

5.5.4 Strain Refreshment

It is wise to "refresh" a strain every so often in order to keep it from degenerating. The procedure is as follows: clone a nicely formed and healthy fruit body from a good crop. Grow a limited amount of spawn and test it under standard growing conditions. If all cultivation tests are satisfying, this clone may be considered as the new parental strain from which the fresh mother cultures will be derived. It is advisable to run the refreshed strain parallel to the existing lines for a while, and only discard the old lines when the fruiting results meet the standards. It does

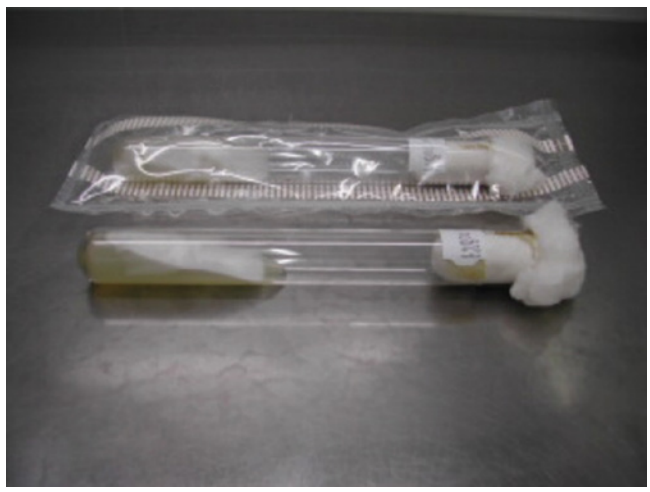

Figure 5.11 Double-packing cultures in fridges limits risks of cross-contamination.

not guarantee a similar leap back in time as the deep-frozen cultures do, but it is a decent alternative. Figure 5.12 illustrates the basics.

Technique: taking a mycelial copy from a mushroom is comparable to the subculture method (see Section 5.6.2): put the mushroom under the laminar flow and tear it in half with the help of two tweezers. Then, flame a sharp tool, for example, a razor blade and cut a 1 cm^3 dice from the fleshy part, without touching the edges of the mushroom. Fresh mushrooms usually contain no bacteria in the fleshy part of the stem. Use tweezers to put it on a petri dish with PDA, malt extract agar (MEA), or another suitable carrier. The mycelium will start growing into the agar from this piece of mushroom. Make a few copies of different parts of the mushroom or from different mushrooms to increase your chances of success.

Notes:

- If all samples are contaminated (usually with bacteria), plate out the mycelial part of the infected culture onto a fresh petri dish. Repeat if needed. Often, a few multiplications later, a pure culture can still be obtained. If the infection is too strong, this technique will not work.
- In some rare cases – for example, it is the only mushroom you have from this species - adding antibiotics to the agar will help you to fight bacterial infections. The antibiotic solution must be micro-filtered and added to the agar after autoclaving. A few examples of usable antibiotics: penicillin (50 units per ml), streptomycin (50 units per ml), tetracycline (30 units per ml) are commonly used this way, either alone or in combination (Wiese et al., 2001).
- The same technique is used for obtaining new species or strains from fruit bodies.

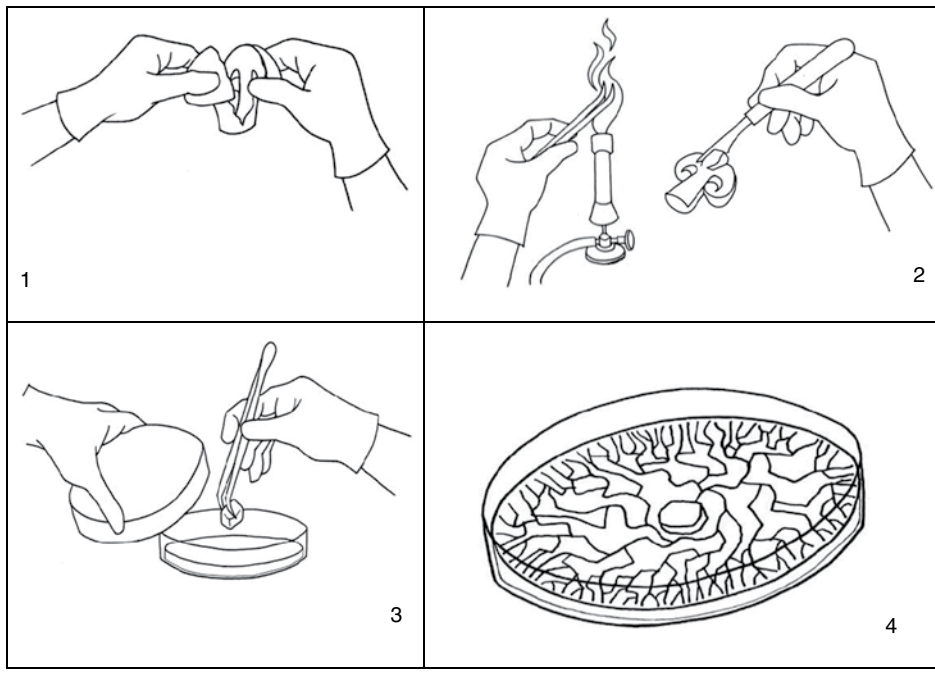

Figure 5.12 Inoculating a mother culture petri dish with mushroom tissue.

1) Tear the mushroom in half with hands or tweezers
2) Flame the tools. Cut 1 cm^2 from the fleshy part of the stem
3) Put this mushroom cube onto the agar
4) Incubate the petri dish

5.6 Production of Mother Cultures and Mother Spawn

5.6.1 Making Agar

Live mycelial mother cultures are typically kept in petri dishes or in test tubes on an agar medium. First, get the correct agar. Food grade agar, as used in the candy industry, is perfectly suited for mushroom growing purposes. There is a multitude of recipes for agar: MEA, potato agar (PA), compost agar (CA), and many others. The chosen recipe is of considerable importance. Adding some ingredients will, at the earliest stage, "train" the mycelium to assimilate the organic molecules that they will be destined to consume.[11] For ligicolous mushrooms, the addition of lignin to the matrix will increase the incubation speed of the mycelium, especially affecting the step from spawn to substrate. For compost mushrooms like *Agaricus bisporus*, the same effect is observed when dried compost phase 2 is added to the matrix. In most countries, the ingredients can also be bought ready-made.

Fungi follow a pattern of growth and development which is much less predictable than for plants and animals. If you plate out mycelium, taken from any source, such as the inside of a mushroom, it is likely that hyphae will emerge and grow on the agar. This means that the hyphae in mushrooms have the same genetic possibilities as the hyphae in a substrate mycelium. All undamaged cells of a viable hypha may initiate a colony, so every cell is totipotent. In other words, fungi do not become fixed into a developmental pathway. This has the consequence that fungi can change their behavior and genotypic expression very fast. As a result, they are extremely flexible and unpredictable creatures.

Technique: the preparation of an agar recipe can be done in a "dirty environment" if so desired. When buying the ingredients ready-made: boil tap water, add agar and other additives at the boiling point and mix well, boil for one to three minutes while stirring. The techniques for making petri dishes and slants are a bit different. Figure 5.13 illustrates the preparation of a PA.

1) *Making petri dishes*. Fill autoclavable bottles up to two-thirds of their volume with the agar medium. Screw the lid loosely on the bottle-neck so as to allow pressure increase and reduction during autoclaving, and prevent the bottles from exploding. Sterilize the bottles using a liquids cycle (see Section 5.8.1). Cool down the autoclave with sterile air. For small volumes, a pressure cooker will do nicely. For large volumes, a small autoclave is better. A two-door autoclave, one door in the dirty rooms, one in the cleanroom's mother spawn department, is ideal. If this is not the case, the autoclave should be located in a *very clean* sluice with exceptionally strict hygiene rules.
2) *Making slants*. Suck 15–20 ml of agar into a syringe and fill a rack of slants. Close the slant with a cotton wool stopper. Cover the rack in aluminum foil to avoid condensation drops turning the filters soaking wet during the autoclaving process. Sterilize using a liquids cycle. Cool down the autoclave with sterile air. Take the hot slants from the autoclave and allow them to cool down in the laminar air flow (LAF), tilted at a 20–30° angle to maximize the free surface.

5.6.2 Subculturing

Subculturing in the mushroom industry is the term referring to the transfer of a fragment of live mycelial tissue on agar onto a "fresh" (i.e., sterile) agar medium.

11 Do not underestimate the mycelium's ability to be trained/selected. A consistent training can completely change a particular mycelium's diet. This way, mycelium has been reported breaking down exceptionally tough materials such as oil residues, baby diapers, car tyres, and heavy sands while still being able to fruit.

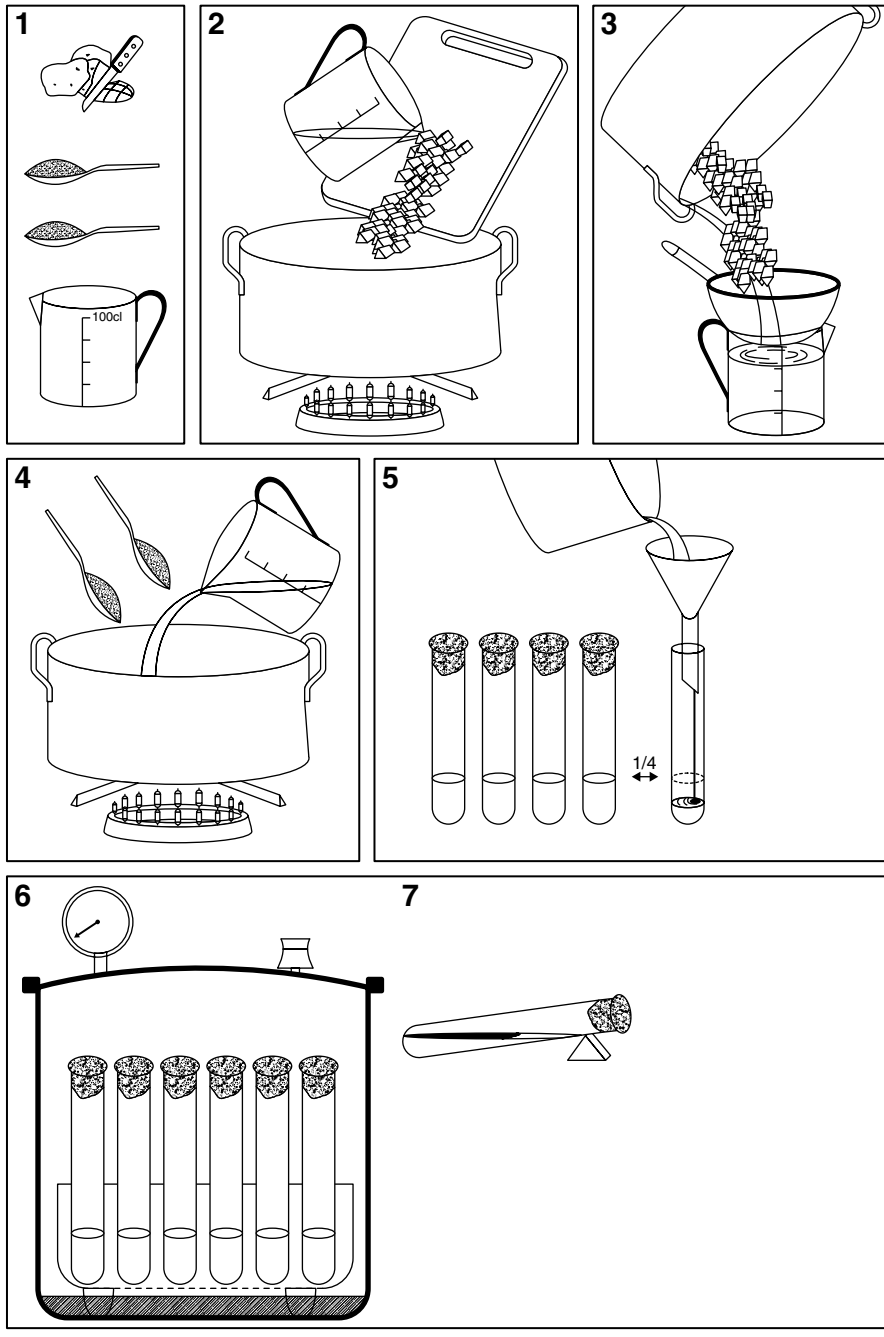

Figure 5.13 Making PA (potato agar) and sterilizing slants.

1) Ingredients: 250 g diced potato, 20 g agar powder, 1 l of water.
2) Boil a pot with the water, dice the potato, and add it to the water. Boil for 20 minutes while stirring occasionally or until the potatoes are tender.
3) Filter the potato broth through two layers of cheesecloth. Save the broth and discard potatoes.
4) Add distilled water to broth bringing volume up to 1 l. Heat the 1 l of broth to a boil. While stirring slowly add agar.
5) Pour agar into slants. A syringe is practical.
6) Sterilize the slants for 20 minutes at 121°C. Cool down with sterile air (e.g., under LAF).
7) Cool down slants at an angle.

Technique: you need a sterile working area, preferably a LAF cabinet or work bench with High Efficiency Particulate Air (HEPA) filters class H13 or H14. Clean the laminar flow hood with a disinfectant, for example 3% bleach, 70% ethanol or a mix based on quaternary ammonium salts at 0.5–2%. Switch on the LAF, put sterile clothes on. Make sure sleeves and gloves overlap at least 8 cm, with the glove on top. Disinfect the gloves. Put tools and materials under the LAF flow hood in a sterile way. Flame the tweezers (Teflon coated, curved) and cut the agar like a cake. Take a piece with the tweezers in one hand, open the second petri dish with the lid facing away from yourself and toward the air flow, and put the piece on top of the fresh agar plate. Carefully close the inoculated petri dish. Do not move your hands between the air flow and *any* petri dish at *any* point. The smallest part, containing at least one living cell, is enough to grow a new petri dish or test tube, but 1 cm^2 and larger is convenient. Tape the edges with Parafilm. Kitchen wrap is a cheap alternative, but it does not have equal breathing capabilities. Incubate the petri dish or slant in a cleanroom at 18–25°C.

When multiplying, you should create parallel descendants (clones) that are then called "lines" (see Figure 5.14). For instance, imagine a fictive mushroom strain called "XYZ" in a mother culture in petri (MCP) dish. Each MCP-XYZ will then give birth to a number of lines 1, 2, 3, … the products can be coded MCP-XYZ-1, MCP-XYZ-2, MCP-XYZ-3, and so on. One of these does not receive a line, as it is a backup. As each of these lines is multiplied further, the suffix -1, -2, or -3 *keeps following* the product code. For example, MCP-XYZ-1 can be multiplied to a number of mother spawns bags MSB-XYZ-1, then to spawn bags SB-XYZ-1, then to substrates SUB-XYZ-1, and eventually to mushrooms. This guarantees that a particular line of a particular strain can later be traced right back to the source. And if there is a problem with one line only, it is easy to discard all products derived from this line.

Notes:

- There can also be two or more steps of mother spawns, for example "A" and "B" (see Figure 5.14).
- If the flow hood sits in a room without sterile overpressure, this room is considered "dirty" and very strict extra measures of disinfection and cleaning must be applied. You should install a sterile overpressure system in this room as quickly as possible. There is no equivalent alternative to a good overpressure unit.

5.6.3 Making Mother Spawn

Mother spawn is produced in small quantities as compared to spawn. Therefore, it is possible to use reusable vessels, such as plastic or glass bottles with cotton wool stoppers, or breathing boxes like the Microbox.[12] Bags are an interesting alternative, at least if they are produced, incubated, and conserved under cleanroom conditions. Whichever the vessel, it is safest to not have the filters touch the grains during this phase of the production.

Technique: the example in Figure 5.15 explains how to make small batches of wheat grain mother spawn. Boil the grain until the inside of the grain is entirely "glassy," that is, all starch has gelatinized. The kernels should still be intact, or the free starch will stick on the filling machines and tools and will cause the grains to become almost impossible to mix. Drain the grains through a sieve and leave them to evaporate till the outside of the kernels is dry. Add 0.25–0.5% chalk as a chemical buffer and 0.7–1% gypsum as a parting agent. Mix gently so as to keep the grains intact. Fill the vessels and insert the stoppers. These could be cotton wool plugs inside a rubber band, for bottles one can use metal lids with cotton wool pads underneath. As a third option, filter bags with filters on top are excellent too. Protect the vessels with an autoclavable foil to avoid the filters getting wet and put them inside the autoclave. Sterilize with a gravity cycle (see Section 5.8.1) and cool down the autoclave with sterile air. One must

12 www.microbox-container.com/

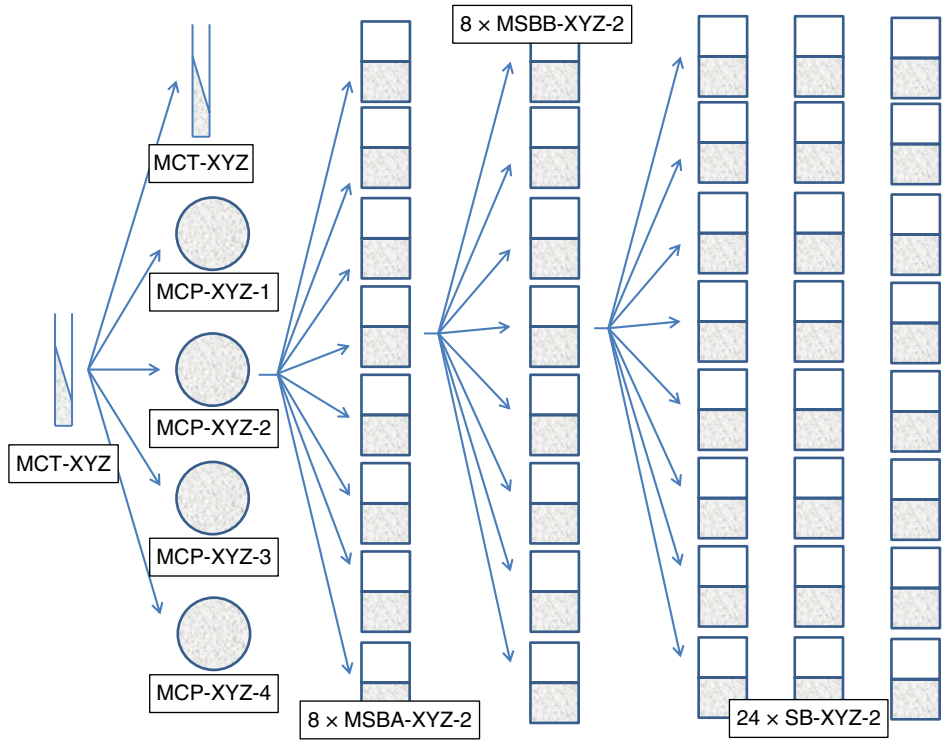

Figure 5.14 Subculturing and the creation of four lines. Example of a four-step breeding program. *Multiplication 1:* subculturing = creation of five mother cultures (1 MCT and 4 MCP). One of these will be kept as backup, the other four become a line parent and are meant for production purposes. *Multiplication 2:* expansion from MCP to eight small mother spawn bags "A" (MSBA). *Multiplication 3:* expansion from one mother spawn bag "A" (MSBA) to eight large mother spawn bags "B" (MSBB). *Multiplication 4:* expansion from one mother spawn bag "B" (MSBB) to 24 spawn bags (SB).

be aware that the time lapse between sterilization and inoculation is an extremely critical phase, because a sterilized vessel can easily be re-infected. The single person handling them must have an insight into air dynamics. All air, including the air in the cleanroom department and air under sterile overpressure, is loaded with particles, a part of which are mold spores and bacteria. The cooling preferably takes place inside a LAF cabinet. The use of wheat is not necessary, many different carriers are suitable as mother spawn substrate. But the rule is that it should be a carrier of good quality, which is easy to incubate, but which also contains enough nutrients to allow for temporary storage.

During inoculation: keep the LAF as empty as possible, organize the workbench meticulously and clean regularly. Don't block the air flow at any point between the filter and the mother cultures or the mother spawn, and again, never move hands, body parts, or non-sterile items between open vessels and the incoming laminar air.

Notes:

- A number of spawn producers choose to grow mother spawn on a powdery recipe on the basis of, for example flour, perlite, and some food additives. Either it is used as such as an inoculum, or this powder is aseptically mixed with sterilized water to obtain a liquefied mother spawn.

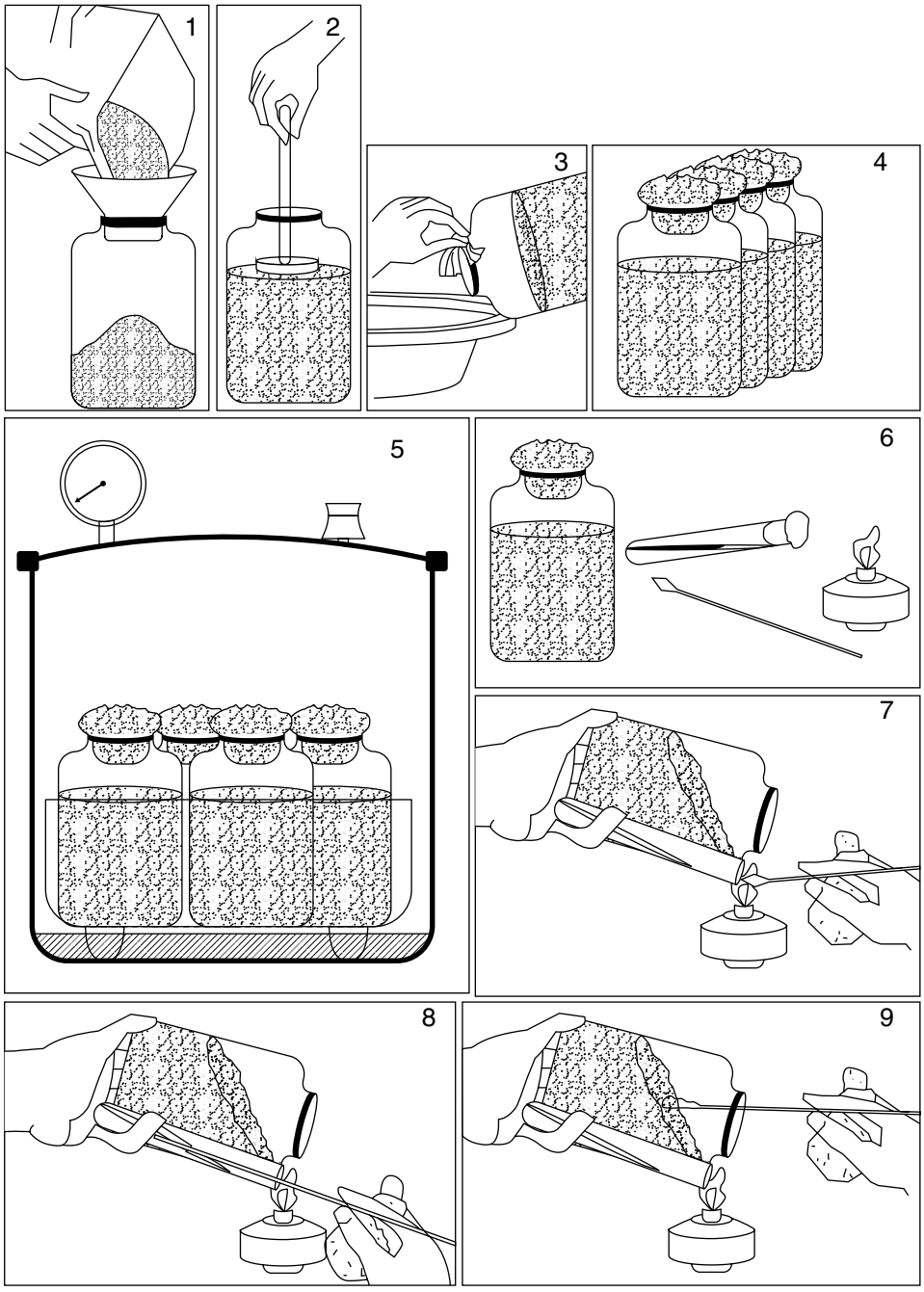

Figure 5.15 Making pre-culture spawn in cleanroom conditions.

1) Filling bottles with boiled grain
2) Press the substrate gently if necessary
3) Wipe the edge clean if necessary
4) Put cotton wool stoppers
5) Sterilizing bottles with gravity cycle and cooling down with sterile air
6) Inoculation tools: inoculation loop or pincer, flame, stock culture

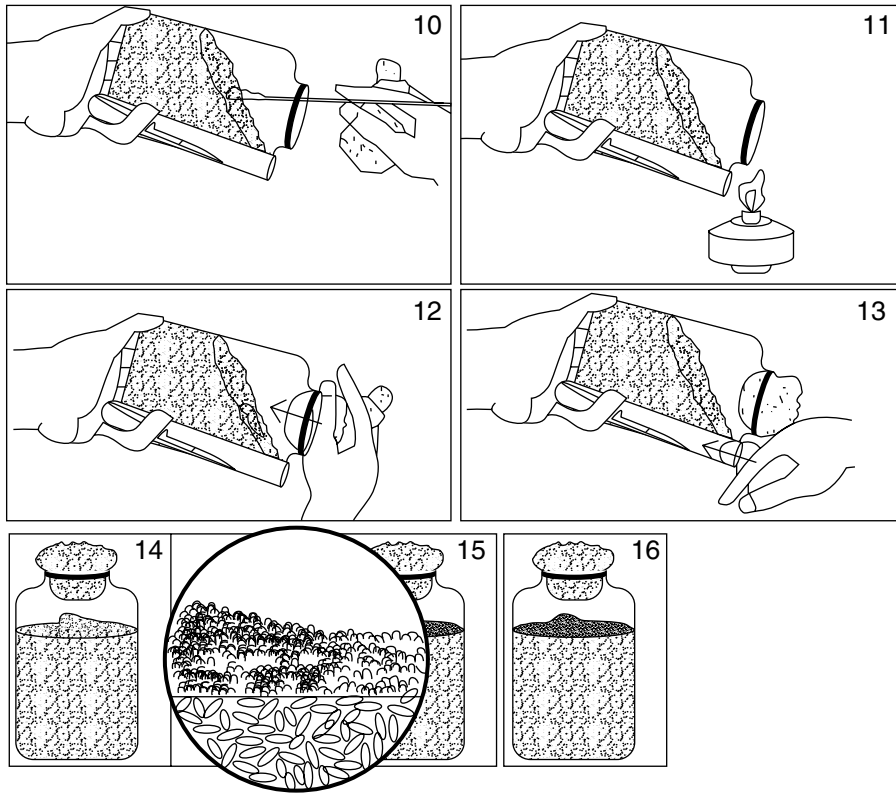

Figure 5.15 Continued

7) Opening both bottle and stock culture. Flaming opening of slant and bottle
8) Gathering 1 cm^2 of stock culture material
9) Flaming the slant opening again
10) Introducing this material into the pre-culture spawn
11) Flaming the bottle neck again
12) Reinserting the bottle stopper
13) Reinserting the slant stopper
14–16) Incubation of pre-culture spawn at 18–25°C

- In the production of liquid spawn, there is no grain phase. All phases up to the end product take place in a liquid medium.

5.6.4 Product Flow and Hygiene Rules in the Mother Spawn Department

A proper mother culture department with near-zero percent losses can only function with a good product flow, proper hygiene rules, and correctly functioning machines.

The most important machines in this area are the autoclave, the LAF, and the overpressure system, with the latter often consisting of several overpressure units. If the mother spawn is produced in bags, a heat sealer is added to this list. Machines are designed to do a job, but humans should check and maintain them regularly. Especially in the case of air filtration units a lot can go wrong and the problems are mostly invisible. Proper tools are needed to check these devices on their accuracy: (1) test petri dishes with a carbon- and nitrogen-rich agar medium, (2) an anemometer to check the LAF's air velocity, and (3) a particle counter to check

the HEPA filters. If the budget is a constraint, leave out the particle counter, but never the other two. If you have no access to a cleanroom or a cleanroom complex, you will have to deal with substantial losses due to mold spores, which will germinate on the bag filters, grow inwards, and sporulate on the inside of the test tube. You can reduce the risk by putting the slant or dish inside a breathing bag and sealing it shut before incubating it. This will stop most infections and it will stop the infections from spreading from one culture to the next. But the only true solution is a good LAF in combination with a performant overpressure system, regular cleaning, and disinfection. However, if you have a proper cleanroom but you do not use it properly, the same losses will occur. There have been examples of mold infections on wet HEPA filters due to steam injection at the end of the autoclaving cycle. HEPA filters are made of paper and are sensitive to physical damage and water damage. Nothing is worse than an inaccurately functioning LAF, because the user feels safe, while the infections are blown right into the product.

The same applies for the refrigerator in which the fully grown petri dishes and test tubes are stored. This room must be reliably clean. It is *very* common for mold infections to occur *inside* a cold store where cold bridges occur and condensation water accumulates, for example, at the door frames and in the corners. The molds will sporulate inside the cold store and these spores will attach themselves on the surface of a petri dish, germinate, and grow toward the inside, regardless of the Parafilm protection. They then form a small colony inside the petri dish and wait for better days to come, or worse, sporulate inside the refrigerator. When this petri dish is used to produce spawn, the end product is spoiled. A perfect mother spawn unit with a contaminated refrigerator cannot deliver top quality spawn.

The production of mother spawn requires the same safety standards as mother cultures. It is strongly advised to implement the production, incubation, and storage of the mother spawn in the same mother culture area, thus forming the "mother spawn department." As shown in Figure 5.16, a fully equipped mother spawn department is in fact a miniature version of a spawn production department, with a similar product flow, but in smaller quantities and with stricter hygiene rules. It consists minimally of:

- Central corridor
- Inoculation room with LAF and sealer
- Storage room with equipment and consumables for the incubation room

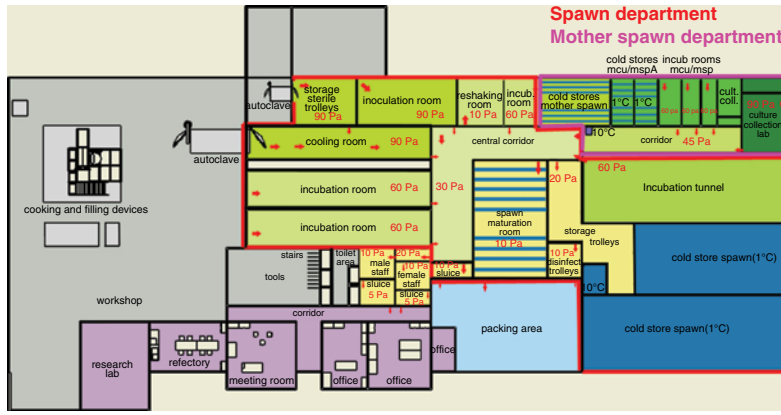

Figure 5.16 Floor plan of a grain spawn lab, depicting overpressure flow and pressure fall. Note the size of the mother spawn department compared to the spawn department.

- Incubation room(s)
- Cold store(s).

A mother spawn area can be restricted, even to one single room, but the rule is the same: extreme precautions must be taken so as to avoid cross-contamination.

People are the most dangerous vector of infection, as they are never 100% free of contamination. There should be as few people as possible entering this part of the cleanroom and only with a fresh aseptic suit. Employees should never be allowed to pass from a dirtier into a cleaner environment, only the other way around. This is comparable to the direction of the air flow: from clean to dirty.

Note:

- As a rule, each line of mother cultures is used to inoculate *the first generation* of mother spawn, also called the grain master or spawn master. These can be used to produce final spawn, but also to inoculate a *second generation* of mother spawn: either in 5-l bags (for larger volumes of spawn) or glass bottles (for smaller volumes).

5.7 Hygiene

Any raw material we wish to use is teeming with life: an abundance of animals, bacteria, fungi, and fungal spores lives within it. These need to be killed so the substrate becomes 100% free of biological life, after which they are inoculated with the selected mushroom strain. But that is not enough. After inoculation, the substrate must be kept from getting infected later on. This is the rule everybody should keep in mind, always: *every environment is heavily infected with microorganisms and fungal spores*. An "environment" can be the air in a room, a water drop, a human, an animal, a floating piece of skin, the surface of a wall, and so on. Of course, not every environment is infected, but the producer should act *as if this were always the case*. That is the only way to maintain cleanliness. A proper level of cleanliness can only be achieved in an artificial environment called a "cleanroom" or "cleanroom department." In such a place, all infection causes are minimized or taken away:

- No taps or drains anywhere
- Smooth walls, ceilings, and floors
- As few people as possible
- As little handling as possible
- People in sterile suits
- Sterile overpressure
- Use of a laminar flow
- Double-door autoclave with aseptic cooling system built-in
- And so on.

In the spawn industry, there are no strict rules and regulations on the cleanliness of such cleanroom departments. It is mostly common sense dictating which rules should be followed. There are, however, abundant examples from other sectors where cleanrooms are used and the rules are similar. The first and most important thing to understand is that all environments are infected. The second most important thing to understand is that all these environments must be cleaned (see Figure 5.17).

The most efficient way of reducing the amount of microorganisms is hygiene. Good hygiene means good cleaning. Whatever the size or production method of the spawn facility: it must be kept meticulously clean. This applies to every part of the cleanroom: the product, the building,

Figure 5.17 Daily cleaning in the spawn cleanroom department.

the people, the bags, the air, the water. But it also applies to the places outside the cleanroom: the offices, the people, the substrate, and compost yards, the surrounding farms, and so on. They all have an effect on the quality of the spawn production site. In a normal situation, the employees are spending at least one-eighth of their time cleaning.

A good cleaning procedure in a cleanroom is one that removes the organic pollution. Such pollution provides nutrition for airborne fungal spores and bacteria. Whenever these develop into a colony in a dead corner of a machine, in a crack in the floor, or behind a silicone seal, they can re-infect the cleanroom from the inside.

The product: this cannot be cleaned. It must be heat-sterilized and kept axenic. All precautions must be taken to shield it from secondary infections: good spawn bags, good bag filters, no punctures.

Bags, bottles, petri dishes: these can be cleaned, but it is better to keep them in cleanrooms under sterile overpressure or wrap them in a breathing bag.

Racks and shelves: cleaning every time they are being reused. No sharp edges.

People: they can only be cleaned to a certain degree. Daily body hygiene is very important. People should wear aseptic clothing whenever entering the cleanroom department: full-body coverall, cap or hairnet, mouth mask, gloves, and cleanroom shoes. There are many optional alternatives, such as showers at work, clean-air showers in the sluice, extra disinfection practices, and so on.

Inoculation room/inoculation machine/bulk vessel: cleaning as much as possible. Either by heat sterilization, chemical cleaning, UV where applicable, water, and soap. In the case of grain spawn: cleaning daily. Machines should be cleaned with water and soap daily if possible, adding some surface disinfectant.

LAF workbench: cleaning many times per day. As much as possible. Thorough cleaning at the end of the working day.

Sterile tools: flaming before every use.

Cooling room/incubation rooms/fridges: cleaning once every 1–4 months, depending on application. Watch out! Condensation forms on cold bridges, which attracts molds. Such zones must be disinfected with a powerful chemical disinfectant. Examples: fridge door joints, badly isolated walls, double walls.

Packing room: cleaning once per week, especially when delivering to producers of sterilized substrates.

Offices, production rooms, substrate and compost production site, and so on: these are not part of the cleanroom, but cleanroom employees pass them daily to get to the cleanroom department, so they should be kept as clean as possible.

5.8 Sterilization, Disinfection, and Filtration

Mushroom spawn must be axenic. That means that it only contains one species and is not contaminated with foreign organisms. Any mistake at this point creates disasters further down the line. There is only one proper way to produce axenic spawn: heat sterilization of the substrate – more specifically with steam – followed by aseptic inoculation. Steam has overwhelming advantages and is universally used in spawn production. When the steam sterilization cycle is completed, the sterilized vessel with the product is cooled down in aseptic conditions. Then, the vessel is inoculated and closed under LAF and moved to an incubation room. After full incubation, the spawn is usually cooled down, packed, and transported, still guaranteed axenic. Keep in mind that sterilizing the substrate is one important step, but maintaining sterility after sterilization is just as important.

There are many ways to kill microorganisms and spores: heat treatment, chemical treatment, mechanical treatment, radiation treatment, and so on. All of these can be efficient, but not all solutions are valuable or practically feasible. The treatments below are the most used techniques in the spawn industry, but the list is not exhaustive.

Equally important to sterilization is making air and water aseptic through filtration. Air and water can be freed of harmful microorganisms by passing them through membranes and filters. These are discussed in Section 5.8.3.

5.8.1 Heat Treatment

Heat treatment is an efficient way of killing all microorganisms in a certain substrate. There are countless applications, but for spawn production, the most important ones are flaming and steam sterilization.

Flaming is the technique where utensils and other objects are held over a flame for a short time. This increases the temperature of a surface locally, killing all life on the particular surface. Flaming is 100% efficient when used correctly. Important: flames create turbulence and disturb the laminar flow locally.

Steam sterilization of a substrate is a very complicated science and is usually underestimated. The proper heat treatment of a substrate starts with choosing a production method: bulk, in bags, liquids, which all require different steam sterilization cycles. But they have a common goal: *every particle of the substrate must be sterile*. To achieve this, each particle must reach a temperature plateau of 121°C or more for a certain time. The amount of time at this plateau depends on the temperature and on the initial spore load of the substrate, which is expressed in a so-called "F0-value." In most spawn substrates, 15 minutes is enough. In bulk and liquids, this is reasonably straightforward as gases are easily evacuated, in spawn bags, bottles, and other containers it is much more complicated because gases get trapped inside the bags (see Figure 5.18).

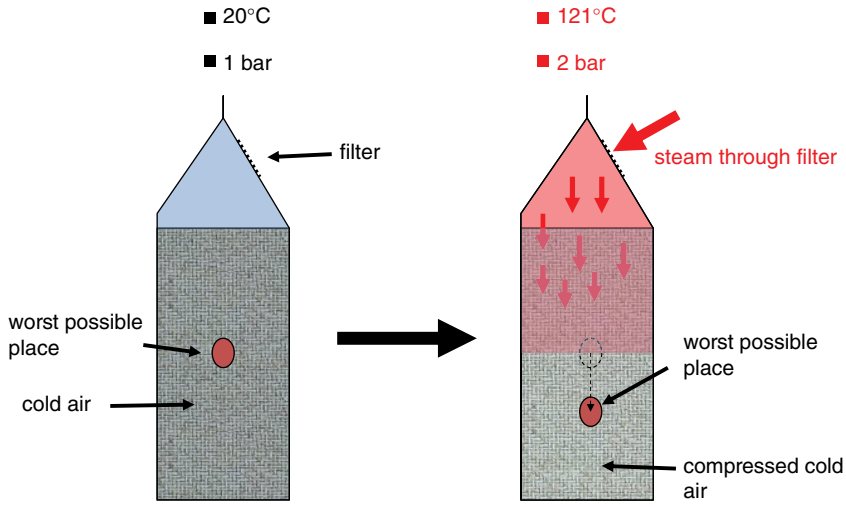

Figure 5.18 Compression of gases in bag during autoclaving.

It is important to keep the sterilization time at 121°C as short as possible. At these temperatures, several chemical reactions take place, such as caramelization and Maillard reactions. These reactions are detrimental for the quality of the spawn: originally available food elements are transformed into indigestible complexes and toxins. The spawn producer should apply the shortest possible sterilization time, while ensuring that all particles are sterilized. The latter can only be determined with the aid of probes,[13] which are inserted in the center of the worst positioned substrate.[14]

There are three main sterilization cycles in the production of spawn: the gravity cycle, the liquids cycle and the vacuum cycle/pulsating cycle. The simplest version of a sterilization cycle is a *gravity cycle*: the pressure and temperature in the autoclave are increased until they reach the sterilization plateau at 121–123°C as displayed in Figure 5.19. This plateau is held stable until all particles are sterile. Then, the temperature and pressure are decreased.

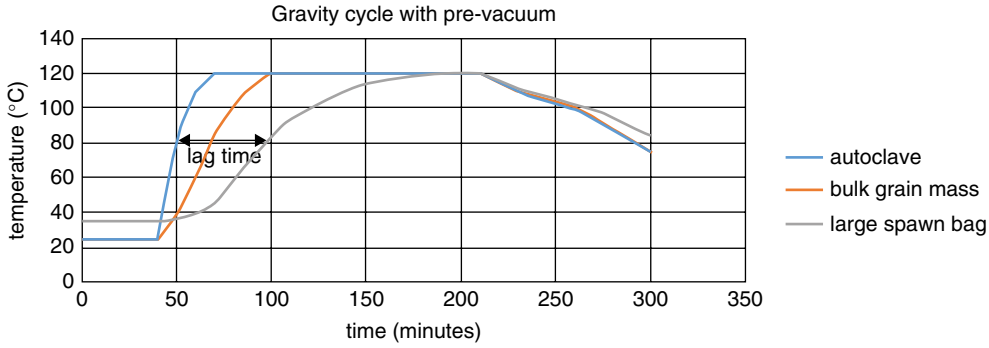

Figure 5.19 Gravity cycle – comparison of different spawn production systems.

13 There are two good types of probes: the first type has a wire and provides real-time temperature readouts, while the other is a cordless temperature USB-probe (e.g., Hobo data logger) which gives readouts after sterilization.
14 As indicated in Figure. 5.18, this worst place is two-thirds down the middle of the most central bag inside the autoclave.

The basic idea behind this cycle is that during the first phase, the air and other gases are replaced by steam by means of gravity and pressure. They must be removed due to the negative effect they have on the sterilization temperature (see Figure 5.20) During replacement, the gases are exhausted through the opened venting valve. This is important, because air and other gases act as insulators, so the temperature of the steam cannot be transferred to the particles. When steam has replaced all gases, the venting valve is closed, allowing the pressure to build up to the plateau. During the plateau, a so-called bleeding valve at the top of the vessel allows for continuous evacuation of gases which form during autoclaving. At the end of the autoclaving cycle, cooling is achieved by slowly releasing the steam through the venting valve, or by allowing the steam to naturally condense inside the vessel. Just before the pressure has reached atmospheric pressure, the venting valve is closed. The volume of the steam, as it condenses and changes into water, will shrink to 1700 times less than its gaseous size. This creates an immense vacuum inside the vessel, which must be replaced with sterile air. So, at the same time when the venting valve is closed, the sterile air inlet valve must be opened. *Important*: if at this point, non-sterile air is allowed into the autoclave, contamination of microorganisms may spoil the entire autoclave.

When sterilizing a grain substrate in a bulk vessel, the simplest version of the gravity cycle can be utilized, as explained previously. The plateau at 121°C must be kept for 40 minutes or less, depending on the substrate recipe. Optional extras that shorten the cycle are: (1) creating a pre-vacuum before pressure increase to eradicate all gases before steam is injected, and (2) using a post-vacuum after cooldown to speed up the cooling process.

When sterilizing grain or substrate spawn in bags – or bottles, boxes, or other recipients – the gravity cycle is not the best option. When steam is injected into the autoclave, it is supposed to gradually replace the air in every part of the substrate during the pressure build up. In an open mix, such as a bulk vessel, this is easy as all pore spaces are open and there are no obstructions anywhere. But in an autoclave filled with bags the situation is totally different.

The bags are being compressed as the steam builds up the pressure, but there is no free exchange of steam and air between the bag and the surrounding space in the autoclave. The gases inside the bags get trapped and act as an insulator: where they prevent the steam from penetrating the substrate, the heat transfer is much less efficient. The heat will only slowly pass through the plastic to reach the substrate particles on the inside. In large bags filled with grain, this process can take as much as 240 minutes. As a result, the temperature inside each bag changes gradually, with the outer layers heating up faster. This temperature gradient causes quality differences inside each bag and an overall quality drop. There are only two possibilities to get rid of this problem: (1) use smaller bags or (2) apply a vacuum sterilization cycle.

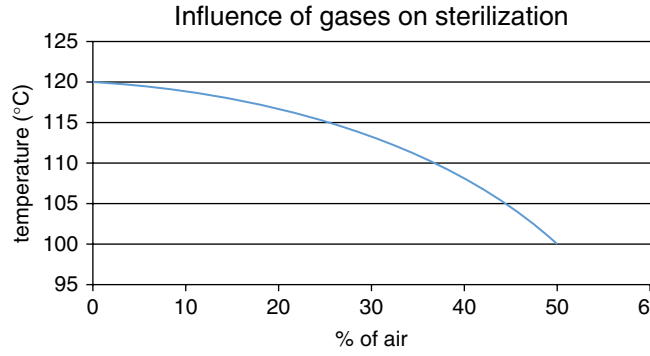

Figure 5.20 Influence of gases on sterilization temperature.

The *liquid cycle* is used to sterilize liquids. Examples are liquid spawn substrates and agar bottles. It is comparable to the gravity cycle, with one difference: during heating and cooling, the temperature must be gradually and slowly increased and decreased. If not, the bottles will burst and/or the liquid will start to boil violently, causing serious problems.

The *vacuum cycle* or *pulsating cycle* (see Figure 5.21) is a rather complicated sterilization cycle. It has been developed specifically for sterilizing porous and bagged goods, such as pre-bagged spawn substrate. The cycle works as follows: the pressure is increased and decreased in a number of pressure pulses, followed by a stable maximum temperature plateau at 121–123°C. During the pulses, the air and gases that are present inside the bags – or bottles, boxes, and so on – are replaced by steam by force. This way, the time for every particle to reach 121°C is shorter and the temperature plateau can be much shorter than during a gravity cycle: 40 minutes or less, depending on the type of substrate and packaging, and positioning of the bags in the autoclave. Without trapped gases, there is no temperature gradient inside the bags. This is very beneficial for the quality of the end product, which reaches similar quality levels as bags produced with bulk sterilization, or as liquids sterilization.

There is no general type of vacuum cycle. The amount of pressure pulses, the height of the pulses, and so on, all parameters depend on the situation. Trial-and-error is the only way to work out an optimally working vacuum sterilization cycle. These are a few important observations:

- The pressure increase through steam supply is the most crucial point of the cycle. If this increase is not fast enough, the steam will not be able to penetrate deep into the bags. That means the steam generator must be able to supply sufficient steam and that the piping, valves, and filters must be operating perfectly.
- If the pressure increases too fast, the bags will compress. If the pressure decreases too fast, the bags will explode.
- The difference between the top and bottom of a pulse must be at least 0.3 bar.
- When using this type of autoclaving cycle, permanent measurements inside the bags during each cycle are essential.

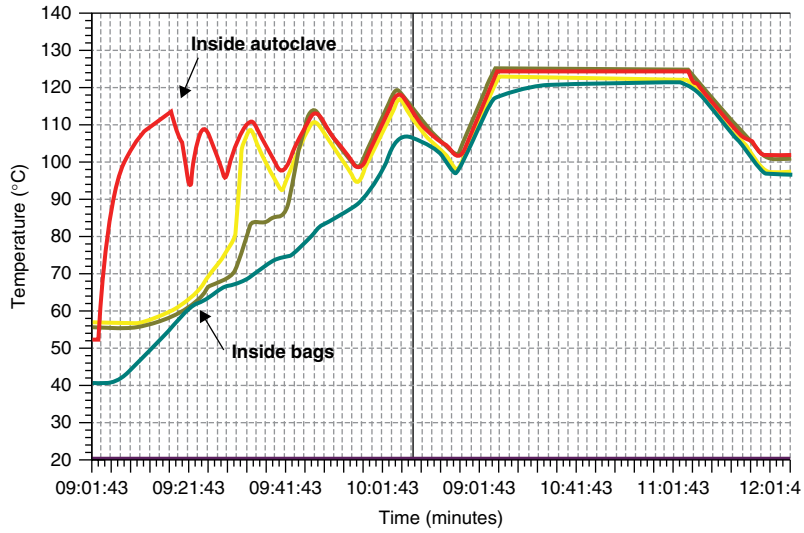

Figure 5.21 Vacuum sterilization cycle – sterilization of porous and bagged goods.

5.8.2 Chemical Treatment

Chemical sterilization and disinfection are mostly used in surface disinfection, and to a lesser degree for sterilization and disinfection through inundation, burning, and vaporization. The efficiency of chemical disinfection is often overestimated.

There are four main types of chemical disinfection in spawn production: burning, soaking, fogging, and surface disinfection, with this last one being the most widely applied.

Some chemicals are meant to be burned. This reaction releases toxic fumes, which kill infections efficiently, but superficially. Examples are formaldehyde and chlorine tablets. Due to the nature of these reactions, the room must contain as few traces of organic materials as possible.

Other products need to be fogged, such as mixtures with formaldehyde and glutaraldehyde as active components, or products based on hydrogen peroxide. These two product groups are highly efficient, but especially when dispersed through the air they are very toxic for all living organisms including humans, as a result of which they cannot be used where people are at work. They are suitable however to disinfect pipework, valves, and confined spaces. Again: due to the nature of these reactions, the room must contain as few traces of organic materials as possible.

The main group of chemical disinfectants, however, are active in the liquid phase. Surface disinfection cannot be seen separate from hygiene. Most products are diluted in water and used on free surfaces inside the cleanroom. The goal is to continuously reduce the number of competing microorganisms. Examples of such products are: hydrogen peroxide (H_2O_2), formaldehyde (CH_2O), glutaraldehyde ($C_5H_8O_2$), quaternary ammonium salts (NR_4^+, e.g., $N(CH_3)_4^+$), sodium hypochlorite (NaOCl = bleach), ethanol (C_2H_5OH), and isopropanol (C_3H_7OH). Just like the airborne chemical disinfectants, these products have only a superficial action. They cannot disinfect a grain kernel to the core, for example.

The activity of surface disinfectants depends on two parameters: the exposure time and the dilution factor. The higher the concentration, the stronger the product, and the longer the exposure time, the more efficient the product. Most surface disinfectants have only a limited range. The author has conducted a very extensive research program (2009, not published) on different surface disinfectants with application for the spawn industry and has come to the following conclusions:

- Most disinfectants are bactericidal after < 15 min at reasonable concentrations
- Most disinfectants are fungicidal after < 15 min at reasonable concentrations
- Almost no disinfectant is sporocidal (fungal spores)
- The few products that were found sporocidal are so toxic to humans that they cannot be used as surface disinfectants without danger.

The most important conclusion is:

- Chemical disinfection is not the key to disinfection in the spawn industry. The habit of regular cleaning (with or even without disinfection products) is far more important.

5.8.3 Mechanical Treatment – Filtration

Not all environments need to be sterilized. Gases and liquids can also be made aseptic through filtration. This is done with filters and membranes. The most important applications in the spawn industry are (1) the use of HEPA filters, (2) the use of membrane, cotton wool, and fibrous filters in breathing bags and other recipients, and (3) the use of membranes in the filtration of air and water in the production of liquid and liquefied spawn.

HEPA filters have large pore spaces, but particles are retained by interception, impact, and diffusion. This is called "depth filtration." A different type of air filtration uses membranes,

where the pore spaces have a certain size and all particles of a larger size are retained. Membrane filtration is also used to produce aseptic water.

A properly functional cleanroom environment is provided with dust-free air under overpressure (see Figure 5.22). The principle behind such an overpressure system is that a permanent flow of excess HEPA- filtered air is supplied to the cleanroom. This puts it in a state of overpressure, causing the air of these rooms to flow toward rooms with lower pressure. This airflow is controlled through ventilation windows and should always flow from zones with high fragility to zones with lower fragility. The overpressure units in a spawn company are provided with HEPA filters with efficiency F9 to H13. A good average is E12. A pressure drop between two rooms of a different fragility level is generally considered safe if it is larger than 15–20 Pa.

Inoculation of mother cultures, mother spawn, and spawn bags should always take place inside a LAF hood. Such a device provides a constant flow of dust-free air, exiting the filters in a laminar way. Most LAFs in the spawn industry use HEPA filters class H13 or H14. The air speed should be 0.45–0.5 m/s to be on the safe side. This should be checked with an anemometer regularly as filters get clogged over time, so the air flow decreases accordingly. The workbench should be a smooth surface, and the airflow should be guided by flaps at the edges of the HEPA filters. 0.8–1 m is the safe working distance of a LAF, but this can be slightly increased if the flow is undisturbed by objects or external influences. Horizontal and vertical flows are equally efficient. For a horizontal LAF the fragile part of the filters are the lower edges, as it can be damaged by cleaning, or accumulate grains during inoculation. Wet filters have the tendency to break or get infected with molds when wet, in which case they must be replaced immediately. Broken filters cannot be patched up or repaired safely (see Figure 5.23).

Breathing bags contain filters. Many of these filters, especially in small spawn bags, are cotton wool pads and wads, which are inserted in the neck of the bag. But many modern spawn bags contain filters which are heat-sealed into place. In these, there are two types: either membrane filtration or depth filtration, with the latter one being more resistant to dehydration. All bag filters allow gas exchange through diffusion. Contaminants and other larger particles are retained.

In the membrane filtration of air and fluids, a fluid passes through a microporous membrane, which retains all particles larger than the pore size of the membrane.

5.8.4 UV Treatment

UV (and ionized air) treatment operates on the principle of creating ionized oxygen atoms, which will then form charged molecules. They also form harmful molecules such as ozone. They

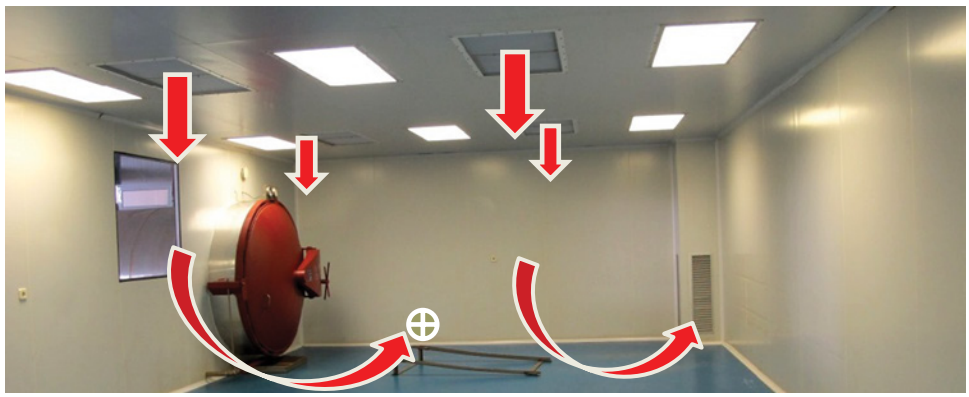

Figure 5.22 Flow of aseptic overpressure air in cooling room.

Figure 5.23 Damaged HEPA filters in need of replacement.

should be scheduled to operate when people are not around. Also, they are only moderately efficient and have a short range, so they only provide a very limited protection (exceptions exist).

5.8.5 Radiation Treatment

Radiation is an extremely efficient way to sterilize objects or substrates. But because of price concerns, it is only used to treat high value objects, such as gamma-sterilized empty spawn bags. These are usually large polyethylene bags (PE) used for bagging bulk grain spawn.

5.9 Substrate Composition

Most cultivated mushroom strains grow on a wide range of substrates. Growing mycelium requires important quantities of carbon (C) and nitrogen (N). Other important elements are manganese, iron, zinc, magnesium, sulfur, potassium, and phosphorus. Mycelium also needs a lot of water during growth. About 42–48% of water in grain spawn is a good average. In the case of liquid spawn, this percentage is much higher, but this is more due to the nature of this spawn than to the limiting requirements.

The C:N ratio (C divided by N) of woodchips of deciduous trees for example can be way over 300:1. However, in most spawn recipes, this ratio will be a lot lower, often between 15:1 and 10:1, which is very beneficial for accelerated mycelial growth. This can be obtained through using richer raw materials such as grains, or a liquid medium with the proper additives. As a result, spawn recipes with a good C:N ratio incubate a great deal faster, decreasing the chance of failure during the spawn production. This same effect is observed when the spawn is used to inoculate compost or mushroom substrates: the leap-off time is shorter. Needless to say, a shorter leap-off time is very beneficial.

There are many spawn substrates: liquid, flour, pelletized flour, grains, seeds, seeds and grains, sawdust, synthesized pellets, and so on. And a great many grains are used: wheat, rye, millet, rice, sorghum, and so on. Most producers use naturally occurring ingredients such as whole grains, milled grains, sawdust, gypsum and chalk, water, and so on. But, for many years, there has been a slight trend evolving toward pelletized and other synthesized spawn types. This spawn does not rule out natural ingredients, in fact it consists mainly of natural ingredients, combined with processed ingredients such as perlite, a vermiculite, powder of these two, and a number of synthesized additives.

Figure 5.24 Millet is an example of a grain type which has a good particle size.

An important thing about spawn is the particle size and shape. An ideal grain spawn for example hosts a large number of small particles of a similar size (see Figure 5.24). The spawn should also be easy to break up. Two things make up most of this consistency: the particles out of which the substrate is built and the density and coherence of the mycelial network. Round-shaped particles break apart easily, especially when the spawn is fresh. This generates a very high amount of equal-sized particles.

The spawn should already contain at least some part of the substrate it is destined to colonize. For example, a spawn destined to inoculate oak logs would ideally contain oak particles such as chips, pellets, or sawdust. This stimulates the mycelium in a certain developmental pathway, producing specific enzymes, which is beneficial to the future colonization. As mycelium is highly adaptive, this can be achieved in a very short time.

5.10 Incubation

The most essential factors contributing to spawn incubation under controlled conditions are temperature, pH, concentration of gases, composition of the medium, inoculation method, growing rooms, and mycelial quality. Environments are typically highly variable and so is the response of the mycelium to environmental factors. Studies on agar indicate that hyphal growth is complex. Initially, hyphal tips elongate exponentially. Subsequently, hyphae elongate linearly, and branches grow exponentially. Growth in heterogeneous conditions results in the mycelium having different parts in different stages of response. Some parts will be responding to available nutrients with exponential growth, others will be static, and others declining. Poorly made spawn will have a heterogeneous aspect: certain zones will be strongly incubated, others will be weak.

Growing mycelium responds to its immediate environment as it expands. Typically, as the first cells colonize a fresh substrate, they will grow fast, elongating and branching exponentially. At a certain point, the nutrients decline and a plateau is reached, during which all remaining space in the substrate is occupied and hyphae grow linearly. After that, the nutrients become

depleted, waste products accumulate in the substrate and the growth declines. Finally, the mycelium dies. In any pioneering colony, four zones are defined: (1) extending zone extracting available nutrients from fresh substrate, (2) productive zone with sharp increase in biomass, (3) stationary zone where ripening, fruiting/sporulating takes place, and (4) aged zone where degeneration and recycling occurs (Figure 5.25).

The idea behind the "ideal" spawn is to use it at the peak of its activity. That means:

- It should contain as little declining mycelium as possible. All particles have just reached the tipping point between the productive zone and the stationary zone.
- The mycelium has fully overgrown and penetrated the particles.
- The spawn is fresh, that is, it has not been stored for a long time.

When the substrate producer makes his own spawn, for example, liquid spawn fermenters in large bottle farms, the spawn does not need to be transported. This is the ideal situation, where the spawn is used fresh and does not need to be cooled at any point. In most situations, though, the spawn is cooled and transported, often over long distances. This interrupts the incubation process.

5.10.1 Temperature

Every mushroom strain has specific temperature requirements. There are two parameters: the survival temperatures and the optimum temperatures. A Shiitake culture for example can resist temperatures from –30–45°C, but the survival depends as much on the temperature as it does on the time of exposure. The mycelium may survive temperatures up to 45°C for a short time, but a longer exposure to 35°C will also kill the mycelium (Tokimoto and Komatsu, 1978). The temperature will have a major effect on certain chemical processes that are associated with the growth of mycelium. Growth speed increases with increasing temperature up to a certain point, after which it goes down again. The apex of this curve is the optimum temperature. In this example of Shiitake, growth occurs between 4 and 35°C, but the optimum lies between 24 and 28°C (Tokimoto and Komatsu, 1982).

Mycelium produces heat during its metabolism. This excess heat is stored inside the substrate and is released into its surroundings. This means that an incubating substrate heats up by itself and because of the optimum temperature curve discussed previously, it will continue

Figure 5.25 Incubating mother culture displaying various zones of incubation.

increasing its metabolism as it heats itself up. The result is that any mycelial volume will overheat and eventually die if it cannot release its heat. Especially during incubation and cooling, it is of crucial importance to monitor and control this temperature. It should be high enough to reach the incubation optimum, but not too high so the temperature increase swings out of control. Incubations rooms should be well ventilated and they should never be completely filled up unless adequate cooling and air refreshment is guaranteed.

Also, inside one bag or bottle, local overheating can occur. For example, where bags are touching on a trolley, the heat release is much more difficult, often resulting in zones where the mycelium dies because of high temperatures. The same problem can occur where the distance to the filter is too great, or in substrate mixes with a low porosity and permeability. It has been observed that where the core of a bag had died from overheating, the edges grew at optimum temperatures.

5.10.2 pH

pH influences growth in a number of ways, most of them being related to enzyme activity during breakdown of the substrate components. Just like with the temperature, this activity goes up to a certain point, reaches an optimum, and then goes down again. Each strain has its optimum pH range. Similarly, solubility of certain components is influenced by pH fluctuations. Many of the cultivated mushrooms have a tendency to lower the pH through their own metabolic activity, thus creating their own selective medium. pH 4 and less is not exceptional in mature substrates.

5.10.3 Gas Concentration

All cultivable mushrooms are aerobic organisms. They require O_2 for their metabolism and produce CO_2. Again, mycelium growth benefits from increasing O_2 and CO_2 levels up to a certain optimum, and then it becomes a disadvantage. Again, too high concentrations are lethal. The ideal concentrations depend on the strain. For many cultivable species, the ideal concentrations of CO_2 lie well over 15% (Zadrazil et al., 1975, 1982) These concentrations are easily achieved inside bags. CO_2 is a heavy gas, it sinks to the bottom of the bags where it will accumulate to over-critical concentrations unless there is a bottom filter. Figure 5.26 illustrates the importance of filter distribution and filter type (Van Nuffel et al., 2016).

Just like with temperature, the metabolism of the fungus will increase with increasing CO_2, so it will produce even more, while consuming more O_2. The limiting factor will in almost all cases not be CO_2, but O_2. A complete depletion of oxygen can occur in the center of the mycelial volume, resulting in non-growth zones at the center of the bag, the furthest away from the filters.

Equally important is the gas exchange inside the spawn volume, which in turn is dictated by the porosity and permeability. A good spawn has a lot of free pore spaces between the particles and these spaces are interconnected. These allow the gases to find their way out from and their way into the substrate by means of diffusion. Obviously, the position and diffusive capacity of the filters in a breathing bag or other vessel is therefore of great importance.

5.10.4 Inoculation Method and Mixing

Every type of inoculation is different. According to their needs and preferences, substrate producers choose to inoculate either with grain spawn, liquid, or liquefied spawn, with sawdust spawn or wooden spawn dowels or sticks. Certain substrates are lightly inoculated,

	HEAD	HIGH	MID	LOW
SD	0	+5%	+5%	+4%
BK	0	+40%	+72%	+90%
UB	0	+26%	+42%	+35%
UT	0	+28%	+51%	+67%

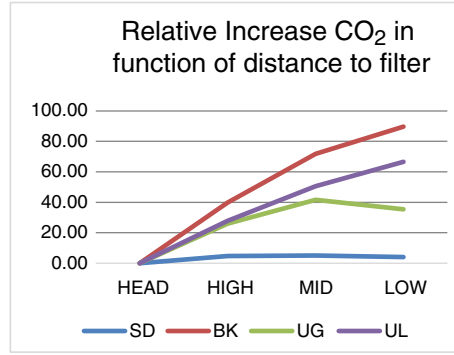

Figure 5.26 Relative increase of $C0_2$ concentration in four spawn bag types in function of distance to headspace at 100% colonization. Head, high, mid, and low indicate the measuring levels inside the bag. The SD bag has filters evenly spread over the whole surface of the bag, the BK, UB, and UT bags have only a top filter strip or patch (Van Nuffel et al., 2016).

others heavily. The used spawn type largely influences the incubation stage of the substrate or compost.

The first important difference is the leap-off time, which defines the lag phase. A substrate inoculated with liquid spawn will have a different growing curve than the same substrate inoculated with grain spawn. A substrate inoculated with liquid spawn starts off slower and then picks up speed at a later stage, finishing more or less at the same time. Liquid spawn grows faster at the end of incubation, so its metabolism will be higher at that point. This means it will require more gas exchange and it will produce more heat.[15]

A second important difference is the inoculation rate. Most spawn producers expand by a factor of 20–60 when inoculating with grain mother spawn and by a factor of 25–200 when inoculating with liquid or liquefied spawn. Heavier inoculation results in more inoculation points and a more even and faster incubation.

The third important factor is the mixing. Incubation is obviously most efficient and fastest if the inoculation points are perfectly distributed throughout the substrate. There are many ways of mixing the substrate after inoculation. In a bulk system, this takes place inside the vessel, or along the way to the bagging machine. In a system of individual bags, it is done just after sealing, for example with a bag tumbler. For the production of liquid spawn there is continuous mixing inside the fermenter.

5.10.5 Incubation Rooms

Incubating spawn is easy, since it is axenic, safely contained and is not meant for fruiting. The ideal spawn incubation rooms are inside the cleanroom department. They don't need to be, but there is much less chance of infection when the bags are placed inside a temperature-controlled room with sterile overpressure. Combined temperature and CO_2 measurement and automated climate control are advisable.

15 A similar effect is observed when a spawn bag is re-shaken. During re-shaking, the mycelium strands are broken into many fragments, each of which gives rise to a new colony after the shaking shock. As all these fragments start to grow at once, there is a sharp increase of metabolism starting 2 days after re-shaking.

5.11 Conservation and Transport

Spawn needs to be shipped and in this case, it needs to be cooled and packed. There are exceptions: liquid spawn, for example, is usually made right where it is being used. It does not need to be cooled down and packed at all. There are also places where the distance between spawn factory and client are negligible.

In theory, spawn can be kept for a very long time in the cold store at 0.5–4°C. But in practice, when it is kept for a long time, its virility declines. So even though some grain spawns can be kept for 5–12 months, they cannot give the same results as a fresh spawn would. Exact speed calculations of this virility decline are not known, but there has been some indication that indicates that this happens very fast: even keeping spawn for a few weeks increases the lag time considerably. Any truly professional spawn company will always sell the spawn fresh and will strongly advise against stockpiling spawn. As a result, in the majority of large-scale operations, spawn is made only on demand and delivered just after incubation. It is the only way to avoid this effect.

When storing spawn in a refrigerator, adjacent bags should not be touching. When they touch, especially those of fast-growing species such as oyster mushroom species, they may start heating up where they touch and will continue incubation inside the cold store. This is most important where the cooling capacity is not sufficient, such as in some refrigerated trucks, but it also occurs in cold stores that do not have enough capacity. Spawn must be packed just before it is shipped and all bags must be completely cold to the core. This takes 5–24 h, depending on the volume of the bag.

When packing the spawn for transport, the bags must be put in boxes that have holes at the sides, to allow for good air circulation. If not, the heat gets trapped inside the boxes and they will start heating up. There should also be space between the boxes on a pallet, for the same reason.

Spawn during transport is fragile. It is cooled down, so the plastics are more brittle. Because of the packaging, there is no visual control. Also, if a bag is slightly damaged, it can easily be infected during transport, as it is transported in non-clean conditions.

When arrived at its destination, the product is often put inside a cold room with doubtful hygiene standards. Examples: the spawn is often stored in the same place as mushrooms or other organic sources that can infect. Or the cold store is not clean. Or the spawn is left packed on the pallet and the bags heat up inside their boxes. The ideal is to dismantle pallets upon arrival and to store boxes in a rack, in a meticulously clean fridge.

When packing mother spawn or mother cultures, extra measures should be taken. These items must be double-packed to minimize external infections and protected from shocks and transport damage.

Producing spawn is complicated and many things can go wrong. In fact, many things do go wrong. As a result, often it is necessary to trace the origins of a spawn all the way back to its source, which can be many months in the past. That is only possible with a proper traceability system. A good traceability system contains for every unit the following information:

- When the recipe was made and who did this
- What the water content was
- How the sterilization went
- Who inoculated and sealed it
- With which culture/mother spawn it was inoculated
- How long and where it was incubated
- When it was packed
- When it was shipped.

There are many ways to keep this information, like a production, inoculation, incubation, and packing note book, a barcode system, a tagging system, and so on. When kept correctly, this information will help with resolving difficult issues like degeneration, bacterial infections, and line instability.

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6

Compost as a Food Base for *Agaricus bisporus*

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6.1 The Place of *Agaricus* strains in Nature

The fungus *Agaricus bisporus* belongs to the Phylum Basidiomycota. Within *Agaricus* you can find this as a heterotroph. Heterotrophic organisms cannot produce carbohydrates from organic products. These microorganisms need to find proteins, fat, sugars, and cellulose in living or dead organic plants via chlorophyll. *Agaricus* can only grow from dead plants and is a saprophyte and "cleaner" of Nature. Nature, as a whole, cleans itself as part of its natural cycle. Bacteria and fungi are at the end of this cleaning process.

For that reason, bacteria are very important in the phase I composting process and, in principle, mushrooms with their mycelium in compost reduce compost's volume and weight by a lot with the production of their fruiting bodies. Fungi, together with bacteria, finally reduce large volumes of dead plants (e.g., peat, compost) so much so (even after mushroom production) that only inorganic product is left over. With this mineralization process, the natural cycle is finally closed. Mushrooms do not contain chlorophyll and therefore they can grow in darkness.

Mushrooms are totally different to plants with chlorophyll because the latter cannot grow without sunlight. Plants with chlorophyll in combination with sunlight work with an assimilation process that produces oxygen and high-value fuels. These high-value fuels are carbohydrates such as sugar and starch.

6.1.1 Bacteria

Bacteria are very small and simple organisms. Some bacteria are so small that even by increasing microscope power up to 500 times it is still hard to see them. Their sizes range from 0.0002 to 0.001 mm. Real bacteria have only one cell that does not contain a nucleus or any plastids. Multiplication/reproduction is by splitting of the cell. Bacteria can absorb food many thousand times more than their own weight per hour. When the environment for bacteria is unfavorable, some protect themselves from change by use of spores. The center of the cell develops a very hard skin and, with this, bacteria can survive during very hot and very cold temperatures, and without food for a long time.

Some bacteria can even survive for 30 minutes at temperatures of 100–120°C. However, most bacteria that can introduce disease in the mushroom process do not have spores and will die at temperatures above 60°C in hours.

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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Bacteria propagate via an asexual manner by splitting of the cell. The halves that are left grow into two adult bacteria. When the environment is optimal for bacteria they can multiply very quickly. In half an hour, there is a new generation.

Bacteria come in many shapes: those that could cause disease in the mushroom process are all rod-shaped. The most important bacteria in the mushroom process that can produce disease are bacteria blotches (*Pseudomonas tolaasii*). There are also very advantageous bacteria such as *Pseudomonas putida*, which is necessary for pinning mycelium to create the fruiting bodies of mushrooms. In the compost process, there are many different types of bacteria and without them it would be not possible to make optimal compost as a good food base for mushroom production. Bacteria are necessary in the composting process for changing ammonia (NH_3) into complicated protein compounds. It is important for mushroom growers to know is that there are three important groups of bacteria (Buth, 2004).

1) Psychrophile group live at 0–30°C;
2) Mesophile group live at 5–43°C;
3) Thermophile group live at 25–45°C minimum and 60–90°C maximum.

Another important group of bacteria are the jet mushrooms (Actinomycetes). These bacteria can be seen after a good phase I and II process in compost. In particular, after optimal phase II processes there are a lot of small white spots: this is the color of Actinomycetes (Figure 6.1). The spores of these bacteria do not grow inside but outside the cell. Actinomycetes live aerobically, which means they require a lot of oxygen and these bacteria are typical cellulose consumers.

6.2 Compost Process Phase I

The production results of mushrooms are very dependent on compost quality. To make compost that can produce high numbers of good quality mushrooms is not an easy job and requires daily optimal concentration on the process. To make a good quality compost you need good

Figure 6.1 After a good phase II process, there are a lot of Actinomycetes that can be seen as white spots on the compost.

quality raw materials. Availability and price of raw materials influence choices made on where to buy them. In principle, you can use all kinds of carbohydrate and nitrogen raw materials to make compost but not all of them are good enough for high production.

It is important that the raw materials are clean and as uniform as possible. In other words, meticulous selection in buying raw materials and care the moment they enter the farm is necessary. Worldwide, there are many kinds of raw materials. The most useful products are wheat straw, rice straw, and horse manure for carbohydrates and chicken, turkey, and horse manure for nitrogen.

There are biological nitrogen raw materials, such as chicken manure, and chemical raw materials, such as urea. Biologically raw materials are products that work less aggressively but take longer to be absorbed than chemical raw materials. Chemical raw materials work more quickly but they are aggressive, however, they help as a kick-starter when there are problems with activity in the process. Chemical raw materials could be good alternatives in times of bird virus epidemics when transport of chicken manure is not allowed. The following lists are examples of useful raw materials.

Useful nitrogen raw materials:

- Urea (46% N)
- Ammonium nitrate (28–32% N)
- Ammonium sulfate (21% N)
- Cottonseed (7–8% N)
- Sunflower (5.9% N)
- Cotton hulls (3% N)
- Cocoa (3% N)
- Chicken manure 2–5% N)
- American hay (1.5–1.9% N)
- Horse manure (1.4–1.7% N)
- Lucerne (1.5% N)
- Fresh pig manure (0.7–0.9% N)

Useful carbohydrate raw materials:

- Straw (e.g., wheat) (+/– 0.5% N)
- Straw covered in horse manure (levels of nitrogen in this depend on feces and urine concentrations)
- American hay (also has 1.5–1.9% N)
- Corncobs
- Corn plants
- Rice straw
- Elephant grass (China/Brazil)

Special raw materials in small amounts:

- Gypsum (5–7% per ton)
  - Protection of structure
  - Reduce greasy compost
  - Controller of pH if you use gypsum with a pH 7 or lower
- Ammonium sulfate (+/– 2 kg per ton):
  - Reduces pH of the compost
  - Increases nitrogen and ammonia nitrogen
  - Keeps activity low
  - Allows for correction of nitrogen later in the process

Before starting the process, you need to have a good composting plan as part of the process. The compost process is a very delicate job and you must not allow mistakes to get in the way of a productive compost. There is an optimal balance between nitrogen and carbohydrates, and for that reason each crop requires a calculation of the right cold start formula to get the right amount of raw materials. Importantly, for a professional process you need to analyze each raw material you need to use.

It is important to know the percentage of nitrogen, ash, and moisture in each different raw material in order to get an optimal balance. The right C/N ratio is very important and seems even more important than the amount of raw materials. Mushroom compost needs 1.6–2% nitrogen from dry organic matter.

In principle, the greater the number of different raw materials used will indicate how much more complicated you make it for yourself, but more numerous raw materials reduce the risk of poor quality raw materials influencing a bad harvest and/or storage quality. However, in principle it is best to not use any poor quality raw material. After performing the right calculations, you know exactly how much you need of each raw material. In a normal standard process, you can produce 4–4.5 tons of fresh compost (finished phase I process) from 1 ton of dry straw.

6.3 Preparing Raw Materials

6.3.1 Prewetting

There are different ways to make good compost. One method is the typical Italian system: mix all raw materials together without any prewetting process. In principle, *prewetting* is not the right word – *presoftening* is a better word for the process. By not using a prewetting period before mixing, the presoftening process happens directly after mixing all raw materials and it takes longer before the compost temperature rises above 80°C.

By using a prewetting process (Figure 6.2) before mixing, the nitrogen source creates a substantial increase in temperature after thorough mixing of all raw materials, including nitrogen sources such as chicken manure. The reason is that a fast increase in compost temperature softens the straw, meaning there is more carbohydrate available for the live microbes; together with nitrogen, this keeps activity low. When not using the prewetting period, the whole fermentation process needs more time because the hard straw has to soften first after mixing all raw materials.

There is a natural hard skin on fresh straw called *cuticula* that protects the straw plant against fungal infections during its growth. This hard layer also makes it more difficult for microbes in the process to attack and for that reason we need some time at optimal conditions, such as the right amount of water and oxygen, to soften.

The prewetting stage aims to make the different raw materials soft for an optimal mix with chicken manure. After prewetting of the straw, chicken manure will bind easily with it. By using different kinds of carbohydrate raw materials, it is important to ensure that these different raw materials have a similar softness before mixing. This helps explain how the more different raw materials a compost facility uses, the more complicated managing the process becomes.

Whether or not a prewet period is used, the production results can still be high. The consequence of not using prewetting is that it takes longer for the compost temperature to get high enough: above 80°C during the fermentation process. A compost facility that doesn't use a prewet period requires more compost moves from one bunker to another.

Figure 6.2 Bale dunking to get a uniform wet start.

Before mixing the raw materials together, some raw materials need preparation. Chicken manure, for example, needs to be cut fine enough so it will connect better to sticky or wet straw. Corncobs also need to be cut before mixing and it is better to prewet them after cutting and before mixing into straw. Prewetting of corncobs makes them soft giving them more uniformity with the soft straw after prewetting (Figure 6.3).

A very important detail is that, during prewetting, corncobs also need oxygen. They should be prewetted on aerated floors ideally, but in case there are no aerated floors, the cobs should be turned daily, or once every 2 days, for better aeration and reducing sensitivity for anaerobic spots. The time for prewetting corncobs depends on corncob hardness and the reaction in the process, but normally corncobs need about 9 days of wetting to be optimally soft.

Figure 6.3 Left: Chicken manure cutter for high volumes. Right: Wetting process for corncobs.

The time taken for wetting wheat straw, for example, depends on the quality of the straw. It will be clear that straw collected during and after a period of dry weather, baled when dry, and protected well against rain during storage will be firmer and need more prewetting time than straw that had too much rain before and during straw pressing in bales. Also, fresh straw at the beginning of a new season normally needs a longer prewetting time than straw from the end of the season. Sometimes there are very wet growing seasons for wheat that can result in very bad conditions when pressing the bales. Straw bales that are pressed when they are too wet suffer a rapid increase in temperature. These bales cannot be stored for a long time for that reason, so it is always necessary to have alternatives.

6.3.2 Mixing and Mixing Systems

It is the case that mixing from different raw materials with tractors and loaders does not produce a good quality compost. To mix raw materials together well there are different mixing systems on the market. In the US, the typical Pannell mixing system is normally used, as shown in part in Figure 6.4, that mixes a sandwich of different raw materials together. The mixing quality is very dependent on how often you mix the same sandwich of raw materials with this machine and even on how you try to set up the different layers of raw materials in the sandwich before mixing.

Another mixing system involves mixing lines produced by several equipment suppliers. Depending on the supplier, the different mixing lines from different suppliers work slightly differently. It is important to know the details of mixing and the biggest influences during mixing on the final quality. For example, during mixing of chicken manure it is not ideal to use water because it can spray chicken manure far too easily to some lower parts of the mixing line where

Figure 6.4 In the US, many compost farms use Pannell mixing machines.

the manure will concentrate too strongly in parts of the straw, and during the mixing process water can also remove too much chicken manure from the straw.

There are simpler and cheaper ways of mixing when the budget available is limited. A simple manure-spraying wagon, as you can see in Figure 6.5, is possible to use but requires more labor time. In second and third world countries, this could be a good option.

Another possibility is to place a nitrogen source such as chicken manure on top of the straw piles and mix it with the Turner minimum of twice through the straw. This is mostly used in the "old" traditional pile composting process (Figure 6.6).

Figure 6.5 A relatively cheap manure mixing wagon.

Figure 6.6 Open air pile composting.

In fact, the system of mixing is not so important, but the way that the system works and the way that the laborers and management handle the mixing process are key to good mushroom production, resulting in a perfect mix of all raw materials

6.3.3 Different Systems of Fermentation

After mixing the raw materials, in principle the fermentation process starts. Fermentation in the mushroom composting process means that nutrients from the raw materials will be more available afterward. What happens in this complicated biological and chemical process is that carbohydrates are attacked by live microbes with millions of bacteria, and the nitrogen source such as chicken manure produces more and more ammonia because of warming up. It is the combination and the right balance of carbohydrate, nitrogen (C/N ration), the right amount of water, plus enough oxygen that stimulate bacteria to work and to raise the temperature. How much the compost temperature increases influences how much more the biological process changes into a chemical process that needs less oxygen than the biological process.

Nitrogen (N) in chicken manure connects to water and changes to NH_4, particular to compost temperatures above 80°C. NH_4 changes to a gaseous state and becomes NH_3. The whole warming up period of the compost up to roughly 65°C is a biological process and this changes slowly to a chemical process. During this fermentation process, oxygen is needed. During the biological process, a lot of fresh air is needed because the live microbes that are active throughout consume a huge amount of oxygen. The chemical process needs less oxygen (think about a small fire at your barbeque that only flames up with very soft blowing on the burning wood).

There are several methods for making good compost. The old traditional system is to use ricks (or piles) (Figure 6.6). But in recent years there has been significant development in composting on aerated floors (Figure 6.7). This idea started in northern Italy and the *tunnel process* was born. In Italy and the Netherlands, growers and researchers have made great progress by research and practical experience in how to make a good fermentation process (phase I) and an easier and better phase II process optimal on aerated floors. Finally, due to pressure from the Dutch government to develop an indoor composting system that makes it possible to produce phase I compost indoors without odor problems, the process of bunker compost was born as well. Today, we see in industrial developed countries that compost facilities working with bunkers or closed tunnel systems provide a very good solution with aerated floor composting.

Countries with strong environmental regulations, such as the Netherlands, produce phase I compost only using indoor facilities (Figure 6.8). Others, such as Canada (Ontario), are encouraged by local governments to use indoor facilities. Costs are high and compost facilities that work only with indoor fermentation try to reduce their costs with high volumes of compost production.

6.3.4 Aerated Floors

There are different systems for using direct compost aeration. One is the typical Dutch system, such as that the CNC (Cooperative Dutch Mushroom Association) calls open floors. The CNC use these floors for phase I and phase II with a continuous aeration process. A big advantage is that, in this system, only fresh air enters the compost for oxygen control. Aeration comes from air circulation that retains energy better in the compost. Also, aeration is continuous, which means it is more uniform. The compost is filled using a cassette and emptied out by a pulling winch (Buth, 2011).

On the aeration floor, there are two different nylon nets for emptying the compost from the tunnel. The first net makes pulling from the other net easier because nylon moving over nylon creates less resistance. The second and much stronger net is a pulling net. In modern winches,

Figure 6.7 (a) An aerated bunker floor; (b) Modern bunker fermentation; and (c) Overhead filling system for filling bunkers as uniformly as possible.

Figure 6.8 Compost hall indoor facility.

Figure 6.9 Softening of straw after wetting on aerated floors.

there is a speed reducer on the pull roll of the winch to ensure the speed of the outgoing compost is consistent, because the pulling roll moves the pulling net continuously around the roll during pulling, which increases the diameter of the net around the roller and this in turn increases the pulling speed if there is no speed reducer.

Another system is spigot floors for bunkers (phase I process) with holes in the bottom that are connected to aeration pipes (Figure 6.9). A fan blows air into a center duct on the front of the bunker, and from there the pipes in the bunker floor are connected. The aeration program goes when the fan stops because there is no return air. Continuous aeration in this system cools down the compost temperature, especially during and at the end of the process. The compost is emptied by loaders. Filling is mostly carried out by an overhead filler but a filling cassette or a filling hopper can also be used to fill bunkers.

In both systems, there are oxygen and compost sensors connected to a process computer (Figure 6.10). The open bottom system has an oxygen sensor and compost sensors close by as well as an air inlet temperature sensor plus air dampers. In modern farms computers control the process from both systems. In both systems, a centrifugal fan is necessary with frequency drivers on each fan too.

It depends on the process used, but in most farms the compost needs to move from one bunker to another or from one tunnel to another after 4 days to improve uniformity and to add some water if necessary. The filling weight is a maximum of 1800 kg per m^2 and the fan needs the capacity to produce 200 m^3 per ton of compost per hour.

6.4 Phase II

6.4.1 What Phase II Means and What Happens in Tunnels During the Process

Phase II is the second step in the composting system. Without phase II it is impossible to grow optimal mushrooms. Phase II is made up of several steps: The first step is *leveling*, which means

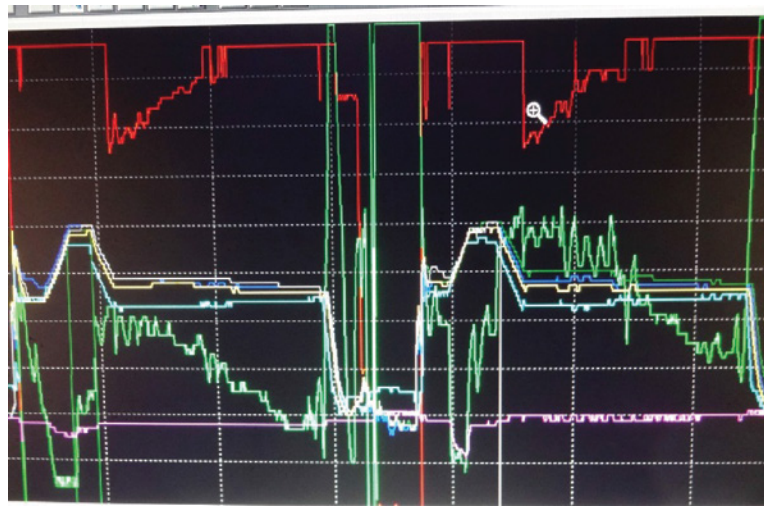

Figure 6.10 Computer control is a must in modern compost facilities and is used in phase I and II of composting.

that with air circulation the system brings the different compost temperatures more in line with each other. During the leveling process the compost will be less active depending on the leveling time and compost quality. The second step is warming up for pasteurization; this warming up process normally happens via use of steam when phase II happens in rooms. Steam is not necessary when the process happens in tunnels. The third step is pasteurization, which is necessary for killing as many undesirable organisms, such as flies, their eggs, larvae, spores from disease-like green molds, bubbles, cobwebs, and other pathogens, as possible.

The minimum temperature for good pasteurization is 56°C and the maximum 60°C. After pasteurization in tunnels for around 6–10 h, the temperature drops down to a conditioning temperature of 48°C: the desirable composting temperature. During this conditioning process, ammonia exchange occurs by beneficial microorganisms to proteins, which are the available nitrogen nutrition for mushrooms near carbohydrates that mycelium and mushrooms find in cellulose, hemicellulose, and lignin from the straw.

After the conditioning process, when all the ammonia has disappeared from the compost, the compost is ready for cooling down for spawning. The right oxygen and aeration in the process is very important for good results. Professional tunnels have oxygen sensors to continue controlling oxygen during the whole phase II process. Normally, the compost is active and needs fresh air to control temperature. However, at each stage of the process it is still important to control oxygen as well as when the compost is less active and so it needs a small amount of fresh air to cool the compost temperature.

Especially during the warming up for pasteurization, it becomes clear that the overall process needs less fresh air, particularly by a lower fan speed than is used during the warming up process. It is important that at no stage of the process should the oxygen be allowed to drop below 12%. The optimal fan speed varies in different stages of the process. During leveling, generally a 100% fan speed is necessary depending on the activity of the compost and range of different compost temperatures that strongly depend on the filling quality. It follows that the amount of compost in the tunnel also has an optimal fan speed.

The maximum amount of compost filling a tunnel is 1400 kg per m^2. The optimal leveling time depends on how uniform and active the compost is. Due to less activity and more uniform

Figure 6.11 Ammonia pump.

compost temperatures the leveling time will be shorter. The most common leveling time is around 12 h. Every extra hour of leveling time makes the available time for good conditioning shorter. By using too great a fan speed during leveling when not necessary, it is possible to remove too much ammonia. The fan speed during the warm up to pasteurization is mostly reduced to warm up the compost. How low the fan speed needs to go depends on fan capacity, amount of compost, and activity of the compost.

The optimal fan speed during pasteurization is 100% to be sure that temperatures everywhere in the tunnel exceed 56°C. The optimal fan speed during cooling down for conditioning is mostly 100% as well to bring the compost temperature uniformly down to 48°C. The fan speed during conditioning is, at the start of the process, normally much lower but each day, the fan speed must be increased a little because the compost changes daily to a softer consistency.

During cooling down for spawning, normally the fan speed is 100% as well to get air everywhere around the compost. Cooling down for spawning in tunnels is only possible when the ammonia (NH_3) levels that can be measured by ammonia pumps is low enough. NH_3 should be below 5 ppm to cool down safely (Figure 6.11).

6.4.2 Systems

There are different systems for a phase II process. There are farms (still in the USA) that work with a one-zone system, which means after phase I the rest of the process happens in the same area, called rooms or houses (Figure 6.12). In a multi-zone system, such as tray farms, phase II also happens in a room but afterward the compost spawns on a spawning line and after spawning the compost incubates in other rooms. In the one-zone system, the grower fills rooms with finished phase I compost semi-automatically using conveyer belts into the rooms. Laborers make the compost as even as possible in the beds and a nylon net on the bottom of the beds is pulled with a winch to move the compost to the end of the beds.

The conveyer belts that bring the compost into the rooms continue in the same position. On the end of the conveyer belts there is another small conveyer that can move at high speed to the left or right to fill the compost over the full width of the beds and laborers finish the process of filling with leveling by hand or a small shovel for a better phase II process. In a multi-zone system, the compost fills the trays in the tray line that growers in such a system use for spawning as well as after the phase II process.

After filling the rooms in both systems, the process starts with leveling that means a fan blows air in the room through air ducts to make the air distribution as even as possible in the

Figure 6.12 One-zone system where all stages of the process happen in the same room.

whole room. After around 24 h (depending on compost activity and grower), the grower starts the warming up process with steam. The air temperature will rapid increase to 56°C for 6 h. The compost temperature will increase as well but more slowly than the air temperature.

The conditions of 6 h at 56° in the air perform a surface pasteurization. As soon all compost sensors read 56°, the compost pasteurization starts. After 6 h at 56° air temperature, the grower stops the steam and cools down the air temperature relatively slowly. As a result of these conditions, the compost temperature will climb to a maximum of around 59–63°C. Because of this slow reduction of air temperature, the compost will drop in temperature as well. As soon the minimum compost temperature is reached, the pasteurization of the compost finishes. In more or less 8–14 days the compost drops down from a 56°C average to a 45°C average.

With this slow drop of compost temperature, all zones of the compost pass the optimal conditioning temperature between 52 and 45°C. This is the temperature range in which thermophilic bacteria work at their optimal level. The most optimal temperature is 48°C in this range. At 48°C, microorganisms in the compost work at their maximum transforming ammonia to protein, the final food base for mushrooms. Between 45 and 52°C this group of microorganisms is more or less as active as thermophilic microorganisms, for example *Humicola* and *Scytalidium* fungi and Actinomycetes.

As soon all ammonia is removed from the compost (testing by laboratory control is an option but an experienced grower can smell it in several spots of the compost and decide whether he or she can cool down the compost temperature). It must not cool down when there is still some ammonia in the compost. It is important to measure NH_3 levels several times during leveling, pasteurization, and conditioning to understand what the process is doing and when you can expect that the compost will be free from ammonia.

During leveling it is advisable to measure three different times and during pasteurization three different times as well. During conditioning, it is advisable to measure NH_3 each day, at the same time each day as far as possible. As soon the NH_3 measures below 5 ppm it is safe to cool down for spawning. If the grower decides not to cool down immediately after all ammonia disappears from the compost, there will be a strong development of Actinomyces in the compost. Actinomyces are also a food base for mycelium but this is not optimal. A lot of it comes

from dust freed during spawning; dust is an allergen and many laborers are very sensitive to it, suffering very severe allergic reactions.

Normally, the fan speed has to increase daily because, during the conditioning process, the compost will get softer and softer. That makes the compost denser and means that less air will move into the compost. The moment of cooling for spawning is always a human-made decision. The compost can cool down in tunnels in a couple of hours to 25°C depending on the outside temperature. Be sure that all compost temperatures are below 30°C before spawning and take care that the temperature does not fall below 25°C. Compost temperatures that are too low after spawning slow down mycelium growth speed.

6.4.3 Tunnel Building for Phase II

For a good phase II process, the tunnel building needs to be of excellent quality. Perfect insulation is a must and the material quality has to be durable. For a tunnel, it is necessary to use a centrifugal fan. Also, a frequency driver is optimal and in fact necessary to control the process. It is important that a fan system sucks steam from the compost out of the tunnel during filling so that the person filling can see exactly how to fill the tunnel. In particular, in cold air warm compost produces more evaporation that look likes steam.

Heating up the air near the filling cassette that blows hot air into the tunnel helps a lot to reduce steam production. In principle, steam for warming up compost temperature after leveling is not necessary because normal compost has enough activity for warming up.

Phase II in tunnels is a better system to use than phase II in rooms. The big difference with phase II taking place in rooms is that the air moves around the compost, and in tunnels the air moves inside the compost. A tunnel process is more secure but also more sensitive. First, it is important that the tunnel is built by professionals and with good materials. When you don't have experience in building tunnels for making mushroom compost it is very risky to try to build them yourself. Many farms in the world have tried to make cheap tunnels with adverse consequences in the process.

A phase II tunnel needs very good insulation of walls and ceilings, a perfect aeration system with the right fan capacity and the right aerated floor. A spigot that is a little too big or too small, or too many/not enough spigots, can create huge problems in the process. There are professional companies with a lot of experience in making phase II tunnels near bunkers and phase I indoor systems. Professional companies normally use stainless steel panels for the ceiling. Wall materials are different depending on the company, varying from stainless steel as well to concrete gas blocks covered by a special coating.

There are different kinds of aerated floor systems for tunnels (Figure 6.13). One type uses spigot floors, which means that over the whole width of the tunnel floor there are pipes in the concrete with air tubes (spigots) that blow air straight into the compost.

Depending on the size of and the number of spigots, plus the fan speed, there are differences in low and high-pressure floors. Another system uses an open floor developed in the Netherlands. This type of floor the tunnel needs nylon nets plus filling and emptying equipment. Filling is done by a filling cassette (Figure 6.14) and emptying by a tunnel winch. The winch pulls the nylon net on a roller and, with movement of the roller the nylon net turns around and onto the roller. With this rolling, the net containing the compost moves out the tunnel.

Because of the high tonnage of compost, these kinds of equipment move using hydraulics. The filling quality of a tunnel is very important and essential for a good phase II process. The filling cassette provides the possibility to fill the compost in different layers above each other so that the compost does not roll down during filling because that creates holes in the compost

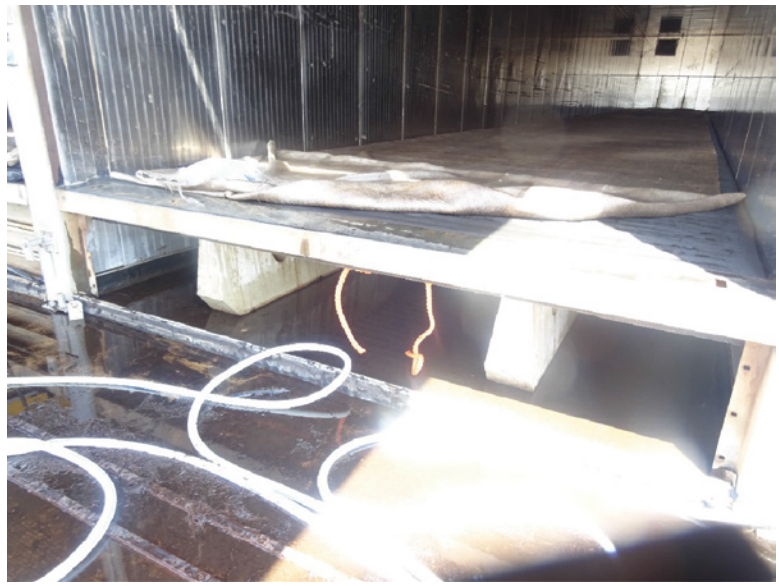

Figure 6.13 Aerated floor from an open tunnel floor.

Figure 6.14 Left: Filling cassette for phase II tunnels. Right: Pulling winch for emptying compost from open tunnel floors with nylon nets.

layer. Air will go in through the lightly filled compost more easily in tunnels (compost holes), which creates less aeration in the rest of the compost resulting in the negative consequence that there is not enough oxygen everywhere in the compost, thus not controlling the compost temperature as well as in more compactly filled compost.

After laborers close the door of the tunnel, the fan needs to start up immediately to blow continuous air into the compost. In principle, the aeration is air circulation, which means that the air that leaves the compost on the top is removed into an air circulation duct that comes again into the fan that again blows the air into the compost. With the necessary oxygen sensor and temperature sensors in the air and the compost, a computer will control how much fresh air must come into the tunnel as well.

During leveling there is a wide range for the air temperature to bring the average compost temperature to an optimal pre-conditioning temperature from 48°C. A great advantage of a tunnel is that all compost sensors are close together in temperature, normally from the end of

leveling from 10–20 h of a 4°C compost temperature difference (depending on filling quality and compost activity). In one-zone rooms there can easily be compost temperature differences of 8–10°C.

The maximum amount of compost that is allowed to fill tunnels is 1400 kg compost per m^2. If the amount of compost is more than 1400 kg per m^2 filling weight it depends on compost structure, free water, and fan capacity as to whether the process can control all details like temperature and oxygen at safe enough levels, but it is definitely the case that the more you fill, the more risky suboptimal control is for the phase II process, which can greatly reduce production.

6.5 Phase III

To decide correctly on how to incubate compost in tunnels is very dependent on the tunnel quality, the whole environment around the tunnels (is there a closed hall with filtering overpressure before and during spawning?), and whether there are enough tunnels for incubation. Other important questions are, how do you fill rooms later with phase III compost? With pressed compost blocks or in bulk? If you plan to fill in bulk, is that with a modern Dutch head filling machine or using the US phase I method explained in this chapter?

Most mushroom farms use a Dutch system to fill phase III compost: in the Netherlands 100% of growers fill phase III compost together with supplement, casing, and CACing plus water on compost and or casing during filling if necessary. In Canada, for example, most growers have a Dutch mushroom farm and they fill with phase III. In Australia, there are several farms with a Dutch system but not all fill with phase III compost, some of them fill with phase II compost in a modern Dutch mushroom farm. In the USA, there are very few modern Dutch mushroom farms but up until now, all of them fill phase II compost.

The reason that filling phase II compost in the US is not available for phase III compost is because not enough tunnels are available. My experience is that all farms that use phase III compost (that is produced with very clean and professional methods in modern Dutch tunnel facilities) have no or very few infections of *Trichoderma* related to farms that fill phase II compost. The major advantages of using phase III compost are many:

- Faster filling schedule in mushroom farms means more batches per room per year.
- Shorter schedule helps to reduce infections from pathogens and flies.
- Filling phase III compost together with casing (Figure 6.15).
- Perfect compost and casing layer is possible by modern head filling machines.
- Using phase III supplement by filling compost.
- Phase III can fill more compost per m^2 than phase II compost that lose +/-10% during incubation.
- Watering phase III compost if necessary during filling (shorter water gift before production).
- The possibility to use compost CACing very uniform with low damage of mycelium in the CACing compost.
- Better compost temperature controls during incubation in tunnels

6.5.1 Spawning

The whole spawning process in tunnels is a very sensitive and secure job. A good hygienic management is a must and the whole process can be very successful when using the correct hygiene rules, but the whole process can be a disaster when the environment is not clean enough. The sensitivity of incubations (spawn runs) in tunnels means that circulating air is

Figure 6.15 Incubation in tunnels that can fill with phase III compost and casing together.

moving continually through the compost during the whole incubation process. When there is some infection during spawning, the circulating air can bring spores everywhere throughout the compost in the tunnel (Figure 6.16). Infection controls with agar plates on compost, spawn, air, and equipment are necessary during the whole spawning process to understand how clean the whole process is. It is important that the spawn storage is built in the tunnel facility to work as cleanly as possible with spawn as well. The advice is to use a cooling store only for spawn that must be separate for spawning!

Some growers use two different spawn strains such as brown and white mushrooms in one tunnel. That is not optimal but is more often necessary. If you need to spawn different strains in one tunnel you need to pull the nylon net from the bottom up to the top of the already filled compost to be sure that the compost with different spawn strains is not connected. The logic in that case is that your tunnel pulling nets need to be long enough to do that and to have enough leftover net for connecting the tunnel winch roll for pulling after the incubation time. Another important detail is that all conveyers and spawn hoppers must be cleaned using clean water before using another strain. However, the best way is to fill only one spawn strain per tunnel.

During spawning it is very important to fill the compost in layers as you also did during filling phase I compost. Filling tunnels in layers makes the filling quality more uniform, which finally gives more uniform compost temperatures and enough oxygen everywhere in the tunnel. The best method during filling is to push the compost sensors in the compost just after arrival at a compost sensor, so that you don't need to walk over the compost after filling because walking over the compost compacts it too much.

Figure 6.16 Filtering overpressure during spawning is necessary to protect the compost during spawning against infections. The plastic air ducts at the top distribute the clean air throughout the whole spawning hall.

6.5.2 Spawn Run

After spawning and after closing the door of the tunnel it is important to start the fan immediately so that the compost receives oxygen. It is important to try to blow the fan at as low as possible a speed so as to not dry out the compost more than necessary. However, the compost temperatures have priority, which means that, if necessary, the fan speed must increase for good compost temperature control of too warm compost temperatures and when the compost temperature range is greater than a grower can accept. The fresh air damper is controlled by computer to maintain a good compost temperature, which means the oxygen level is normally no problem and because active compost needs more fresh air to control the temperature, this means less CO_2 from outside enters the process. Mycelium growth is normally better at a high CO_2 level but there are limits. In normal active compost, too high levels of CO_2 are mostly not a problem but in case the compost activity is not really high it may be necessary to work with a minimum fresh air level from 5–10%, depending on the outside conditions.

During the first week of incubation the fan speed can normally be lower than during the second week, because after 7 days of incubation the compost will become more active daily. During the first week, working with a higher air temperature is allowed (depending on the amount of compost and activity), a 21–23°C air temperature during the first incubation week is usual. During the second week of incubation the fan speed must increase daily and the air temperature will drop a little per day. Modern tunnels have a dry and a wet bulb thermometer. It is important to use the wet bulb thermometer as air temperature during incubation should have fewer air temperature waves (see Figure 6.17).

The incubation time is very dependent on the schedule from the mushroom farm. Most tunnel facilities use 14–17 days of incubation, but that is a tight schedule and depends on

Figure 6.17 (System under phase II). There are different methods of incubation. In plastic bags, blocks, and even in modern shelves; all of them need to be cased later.

availability of tunnels. Speed spawn, for example, can incubate a good quality compost so quickly that it is possible to case compost after 11–12 days of incubation.

After the incubation time in the tunnel it is advisable to cool the compost down a little in temperatures during the summer period to below 22°C, and when the compost has to be transported over a long distance to the mushroom farm it is necessary to cool down enough so as to not result in health and CO_2 problems in the compost. It is advisable to cool the average compost temperature below 19°C in such cases. Modern tunnel winches have a speed reduction system to reduce the pulling speed during tunnel emptying. By not using a speed reduction system, the net on the roll would stretch and slowly become longer in diameter.

To have the same speed of pulling is very necessary when the compost is supplemented during tunnel emptying. In case the compost is too dry, the best way to add water is spraying via a water pipe in the winch just where the compost sprays out the winch axes with teeth while it falls on the conveyer belt.

Supplementing of phase III compost is only possible when the compost is completely incubated, if there is no *Trichoderma* infection in the farm, and if the compost temperature can be controlled easily, plus it is necessary to mix supplements as uniformly as possible. Using the right professional supplements can increase production by 5–8 kg per m^2. Filling trucks for transporting the phase III compost to the mushroom farm is a secure job. The best method is by filling the truck in layers, which means the truck must move forward and backward during filling to get optimal compost uniformity.

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7

Casing Materials and Techniques in *Agaricus bisporus* Cultivation

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7.1 General Aspects of Casing and Fruiting

The cultivation technology of *Agaricus bisporus* (Lange) Imbach involves the following stages: preparation of a pasteurized selective substrate (compost), preparation of the spawn on cereal grains, inoculation of the spawn into the compost, and, finally, cultivation in growing rooms (incubation, casing, prefruiting, induction of the fruiting, and harvest).

In commercial cultivation of *Agaricus bisporus*, fructification occurs in the casing layer, material used as a top covering of the compost usually after the substrate is colonized by mushroom mycelium, to induce the transition from vegetative to reproductive growth (Figures 7.1 and 7.2). The casing layer plays a very important role because it conditions the stage of fructification. A compost that is completely colonized by mycelium will not on its own produce mushrooms. It is therefore necessary to modify the compost to initiate fructification. Although the role of casing layers has not been precisely defined, it must have particular physical, chemical, and microbiological properties that determine their function. The ecological modification which implies the beginning of this fructification takes place in this layer and represents the basis of the interest in mushroom as a commercial crop. Full fructification is encouraged so that mushroom cultivation is made as profitable as possible. Moreover, casing layers, made of materials of a very diverse nature, are an important source of variation in terms of the yield, quality, and uniformity of commercial cropping.

The casing layer fulfills several functions (Bazerque and Laborde, 1975; Visscher, 1988); among others, it:

- constitutes the physical support of the emerging carpophores,
- contributes to the maintenance of a moist microclimate to help feed the mycelium and supports the formation of primordia,
- supplies water for the growth and development of mycelium and fruit bodies, providing a reservoir for the maturing mushrooms and supplementing the water provided by the compost,
- facilitates the transport of dissolved nutrients to the carpophores,
- acts as a suitable medium for the development of mushroom mycelium and bacteria that stimulate fructification,
- provides the mycelium with a suitably aerated environment, facilitating gas interchanges,

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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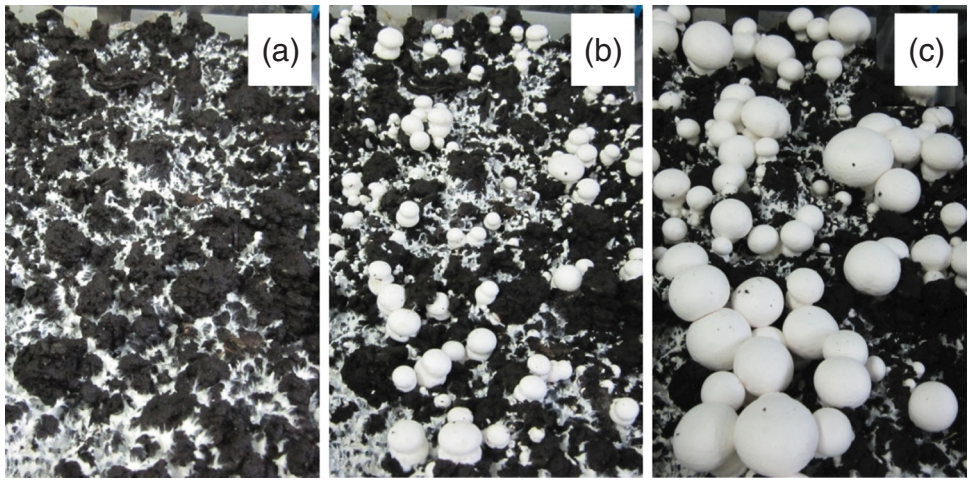

Figure 7.1 Fruiting of mushrooms on a peat-based casing (interval A–B: 5 days; interval B–C: 2 days).

Figure 7.2 Compost (lower) and casing (upper) layers in mushroom production.

- protects the compost surface from drying out and against too rapid disappearance of useful metabolic products,
- has a protective action against antagonistic microflora and induces modifications of the compost microflora (Gillmann et al., 1994),
- provides an environment of low osmotic value unlike compost, whose osmotic value is too high for mushrooms,
- provides a zone where ion exchange may take place (Stoller, 1952b; Kurtzman, 1996a).

In short, casing contains and favors the factors that induce fructification. Many factors are involved in the process. The initiation and subsequent development of the carpophores of *Agaricus bisporus* depend not only on the genetic capacity of the mycelium to fructify but also on physical, environmental, chemical, nutritional, and microbiological factors (Table 7.1). A complex interaction occurs between these factors, impeding the establishment of a definitive theory to fully explain the phenomenon of fruiting. Complete reviews of factors involved in

Table 7.1 Interrelated factors involved in mushroom fruiting.

Factors of Fruiting	Main Aspects to Consider
Physical	Aspects related to structure of materials: • Texture • Density and porosity • Water-holding capacity and water relations • Size and stability of aggregates
Environmental	Aspects related to climate conditions in the growing rooms (environmental transition): • Compost and air temperature • Relative humidity gradient • Aeration (carbon dioxide concentration and inhibitory volatile organic compounds)
Chemical and nutritional	pH Soluble salts (electrical conductivity, osmotic pressure) Chemical reactions in the casing layer Role of calcium carbonate Cation exchange capacity Different nutritional situation between compost and casing Readily decomposable organic matter content
Microbiological	Microbiota present in the casing layer Role of stimulatory bacterial populations (mainly *Pseudomonas* spp.)
Genetics	Genetic heritage of mushroom strains (fruiting ability)

Agaricus bisporus fructification were carried out by Couvy (1972, 1973) and Pardo-Giménez et al. (2002a, 2002b).

It is well-known that some physical and chemical properties, such as porosity, water-holding capacity, salinity, and pH, can affect both the vegetative and reproductive growth of mushrooms. In fact, for an adequate profitability in commercial cultivation, the casing material must fulfill certain conditions (Hayes, 1981; Stamets and Chilton, 1983a; Flegg and Wood, 1985; Rainey et al., 1987; Visscher, 1988). For example, it should:

- be sufficiently resistant and deep enough to provide adequate support for mushroom growth,
- have a high capacity to absorb and release water, be able to withstand frequent irrigation without serious structural breakdown and possess a structure and porosity which permits good permeability for water and gases,
- have a low nutritional value and sufficiently low salt concentration to minimize any water deficit which would be unfavorable to the initiation of growth,
- be of neutral or slightly alkaline pH,
- contain calcium carbonate, mainly for its buffering effect against changes in pH,
- have a high cationic exchange capacity,
- have a low magnesium content and low levels of other toxic elements,
- be free of parasites and competitors.

In addition to the practical experience of growers, detailed knowledge of the characteristics of the casing layer involves the need for laboratory tests for the main parameters associated with these features. This is of great importance, especially in preparation of mixtures of materials and in the subsequent handling of the different operations in the growing room, particularly with regard to the timing of the crop cycle management, watering, ruffling, and environmental control. The main parameters and methods used in the analysis of casing soils and their components are presented in Table 7.2.

Table 7.2 Main parameters and methods used in the analysis of casing soils and their components.

Parameter	Method	Standard/Reference
pH	Potentiometric	European Standard EN 13037:1999 (AENOR 2001a)
Moisture content	Gravimetric (drying to a constant weight)	European Standard EN 13040:2007 (AENOR 2008a)
Electrical conductivity	Conductimetric	European Standard EN 13038:1999 (AENOR 2001b)
Total N content	Kjeldahl	European Standard EN 13654–1:2001 (AENOR 2002)FOSS, 2003
Organic matter and ash	Thermogravimetric (calcination)	European Standard EN 13039:1999 (AENOR 2001c)
Particle real density	Calculation from ash content	European Standard EN 13041:1999 (AENOR 2001d)
Bulk density (fresh)	Weight of certain volume of material after compaction	European Standard EN 13040:2007 (AENOR 2008a)
Bulk density (dry)	Calculation from fresh bulk density and moisture content	European Standard EN 13040:2007 (AENOR 2008a)
Total porosity	Calculation from dry bulk density and real density	European Standard EN 13041:1999 (AENOR 2001d)
Particle size distribution	Mechanic sieving	European Standard EN 15428:2007 (AENOR 2008b)
Texture	Densimeter	Bouyoucos, 1962 MAPA, 1994c
Water-holding capacity	Saturation and drainage	Ansorena, 1994
Water release curve	Suction at different water tensions	De Boodt et al., 1974 Ansorena, 1994
Permeability	Measuring of percolation rate	Rangel et al., 1996
Cation exchange capacity	Barium chloride	ISO 11260:1994; UNE 77300:1996 (AENOR 1996) SISS, 1985
Cation exchange capacity	Barium acetate	Harada and Inoko, 1980
Buffering capacity	Change of pH after addition of HCl aliquots	Rainey et al., 1987
Active lime	Ammonium oxalate (Bernard calcimeter)	MAPA, 1994b
Total carbonates	Hydrochloric acid (Bernard calcimeter or Scheibler apparatus)	UNE 103–200–93 (AENOR 1993) MAPA, 1994a ISO 10693:1995; UNE 77317:2001 (AENOR 2001e)
Pathogenic nematodes	Baermann funnel	Agrios, 2005
Mites	Berlèse-Tullgren funnel	Brady, 1969 Krantz, 1986
Competitor molds	Growth in petri dish	Tello et al., 1991

7.2 Casing Materials

Many materials, alone or in combination, have been used as casing both at commercial and experimental level, although only very few have proved to be of practical application. Some of them have only been used at the experimental level to study some of their characteristics or certain aspects of fructification, while many others have been discarded for diverse reasons. The behavior of the material as regards to quality and yield of the resulting mushrooms, its availability, and price, are determining factors when choosing a particular casing material.

Among the numerous materials which have been used are materials of a mineral origin (natural or heat treated and industrial wastes), of vegetal origin (natural or transformed), and even synthetic materials. Of these, peat constitutes the most widely used material as casing for mushroom cultivation throughout the world. Its water-holding capacity and structural properties are widely accepted as ideal for the purposes of casing (Yeo and Hayes, 1979). However, problems associated with its use, especially as regards its availability, the depletion of reserves, and the alteration of ecosystems, have led to the search for alternative materials (Price, 1991). In addition to peat moss, natural materials of mineral origin, such as soil, gravel, and calcium carbonate in different forms, and spent *Agaricus bisporus* compost are the most common (Pardo et al., 1999). Mineral materials are usually combined with different organic substrates, mainly peats, which act as structural and water-holding correctors.

Any material which is to be considered as an alternative to peat for use in mushroom casing should have the following properties: similar performance characteristics at least equal to peat, competitive cost, stable quality, continuity of supply, freedom from pests and diseases, and ease of handling (Border, 1993).

A list of materials that have been used or evaluated as ingredients of casing layers in mushroom growing, either commercially or experimentally, is presented next. Each one is accompanied by a selection of bibliographic references. Previous reviews of materials were carried out by Pardo et al. (1999), Poppe (2000), and Jarial et al. (2005b).

7.2.1 Materials of Mineral Origin

7.2.1.1 Natural Materials

- Different soil types (Pizer and Leaver, 1947; Pizer, 1950; Stoller, 1952b; Reeve et al., 1960; Edwards, 1974; Hayes, 1978).
  - Clay and clayey soil (Bels-Koning, 1950; De Kleermaeker, 1953; Edwards and Flegg, 1953, 1954; Hayes and Shandilya, 1977; Edwards, 1978; Visscher, 1982, 1988; Stamets and Chilton, 1983a; Shandilya, 1989; Khanna et al., 1995; Kurtzman, 1995a, 1995c; Maas, 2003).
  - Sand and sandy soil (Edwards and Flegg, 1954; Stamets and Chilton, 1983a; Khanna et al., 1995; Angrish et al., 2003; Toker et al., 2007; Yilmaz et al., 2007; Simsek et al., 2008).
  - Clay loam soil (Edwards and Flegg, 1953; Nair and Bradley, 1981).
  - Fargo silty clay soil (Kurtzman 1995a, 1995b, 1997, 1999).
  - Loam soil (Hayes and Shandilya, 1977; Hayes, 1978; Stamets and Chilton, 1983a; Eicker and van Greuning, 1989; Khanna et al., 1995).
  - Marl, limestone, marlstone (Bels-Koning, 1950; Reeve et al., 1960; Hayes and Shandilya, 1977; Hayes, 1978; Hayes et al., 1978; Stamets and Chilton, 1983a; Visscher, 1988; Kurtzman, 1995a, 1995b; Maas, 2003).
  - Diatomite, diatomaceous earth material, charro (Reeve et al., 1960, Huerta et al., 2001).
  - Chalk (Reeve et al., 1960; Allen, 1976; Stamets and Chilton, 1983a; Rainey et al., 1987; Noble and Gaze, 1995; Stamets, 2000).

  - Garden soil (Bels-Koning, 1950; Rao and Block, 1962; Vijay et al., 1987; Singh et al., 2000; Om et al., 2008).
  - Forest soil, forest litter (Hayes and Shandilya, 1977; Shandilya, 1978; Vijay et al., 1987; Colak et al., 2007; Toker et al., 2007; Yilmaz et al., 2007).
  - Muck soil (Reeve et al., 1960).
  - Vertisol soil, black swelling clay soil with traces of low-grade coal (Van Jaarsveld and Korsten, 2008).
- Gypsum (Stoller, 1952a, 1979a; Atkins, 1979; Visscher, 1988; Stamets, 2000).
- Gravel (Reeve et al., 1960; Gardner and Davies, 1962; Hayes and Shandilya, 1977; Hayes et al., 1978; Clancy and Horton, 1981; Garcha and Sekhon, 1981; Kurtzmann, 1995a, 1997, 1999).
  - Gravel made of volcanic ash (Kurtzman, 2000).
  - Free of sand aquarium gravel (Kurtzman, 1999).
- Tuffeau (Bazerque and Laborde, 1976; Moguedet and Kaeffer, 1991).
- Crushed basalt rock ("blue metal") (Clancy and Horton, 1981).
- Pumice soil (Rainey et al., 1987).
- Dolomite (Stamets and Chilton, 1983a; Kurtzman, 1991, 1997).
- Stone grindigs (Maas, 2003).
- Bentonite (Beyer, 2004).
- Zeolite (Noble et al., 2003; Beyer, 2004).

7.2.1.2 Processed Materials and Waste Products

- Vermiculite (De Kleermaeker, 1953; Edwards and Flegg, 1953, 1954; Barnard, 1974; Eicker and van Greuning, 1989; Verbeke and Overstyns, 1991; Noble and Gaze, 1995; Fermor et al., 2000; Stamets, 2000; Noble et al., 2003).
- Perlite (Gardner and Davies, 1962; Barnard, 1974; Lelley et al., 1979; Colak, 2004; Colak et al., 2007; Baysal et al., 2007; Peker et al., 2007; Toker et al., 2007; Yigitbasi et al., 2007; Yilmaz et al., 2007).
- Rockwool (Visscher, 1982, 1988; Noble and Gaze, 1995; Noble et al., 2003).
- Capogro®, mineral wool product spun from molten rock (Wuest and Beyer, 1996).
- Peatwool®. Mineral fibers, made from molten rock, plus 25% (v/v) sphagnum peat moss (Wuest and Beyer, 1996).
- Used horticultural rockwool (Fermor et al., 2000).
- Expanded aluminum silicate (Reeve et al., 1960).
- Turface, flakes of calcined montmorillonite clay (Kurtzman, 1995a, 1995b, 1996b, 1997, 1999).
- Crumbled bricks, brick chips (Bels-Koning, 1950; Edwards and Flegg, 1953; Edwards, 1974).
- Granulated rockwool slabs, waste product of the glasshouse industry (Noble and Gaze 1995; Noble and Dobrovin-Pennington, 2001).
- Pieces of mosaic (Colak et al., 2007; Peker et al., 2007; Toker et al., 2007; Yilmaz et al., 2007; Simsek et al., 2008).

7.2.2 Materials of Vegetal Origin

7.2.2.1 Natural Materials

- Peats of different types and origin (Stoller, 1952a; Edwards and Flegg, 1954; Reeve et al., 1960; Rao and Block, 1962; Edwards, 1974; Ganney and Richardson, 1974; Caron, 1987; Visscher, 1988; Bellmont, 2005; Baysal et al., 2007; Pardo-Giménez et al., 2012).
- Sedge peat (Clancy and Horton, 1981).

- Pine bark (Allen, 1976; Rainey et al., 1987; Visscher, 1988; Shandilya, 1989; Pardo-Giménez et al., 2012).
- Sawdust (Allen, 1976; Hayes et al., 1978; Nair and Bradley, 1981).
- Gum sawdust (*Eucalyptus* spp.) (Eicker and van Greuning, 1989).
- Pine sawdust (*Pinus* spp.) (Eicker and van Greuning, 1989).
- Coconut coir pith (Border, 1993; Labuschagne et al., 1995; Gupta, 1997; Fermor et al., 2000; Kurtzman, 2000; Noble and Dobrovin-Pennington, 2001; Noble et al., 2003; Dhar et al., 2003, 2006; Suman and Paliyal, 2004; Rangel et al., 2006; Afewerki and Korsten, 2008; Van Jaarsveld and Korsten, 2008; Pardo and Pardo, 2008; Pardo-Giménez et al., 2012; Chandra et al., 2014).
- Rice husk/rice hulls (Nair, 1976; Clancy and Horton, 1981; Rangel et al., 1996; Cai et al., 2002, 2008).
- Barley fines (Hayes et al., 1978).
- Poppy straw (Clancy and Horton, 1981).
- Cottonseed meal (Nair et al., 1993).
- Soyafodder (Van Jaarsveld and Korsten, 2008).
- Lignite, brown coal. Soft brown combustible sedimentary rock that is formed from naturally compressed peat (Fermor et al., 2000; Noble et al., 2003).
- Wood charcoal (Noble et al., 2003).
- Anthracite coal (Noble et al., 2003).

7.2.2.2 Processed Materials and Waste Products

- Wood and bark wastes:
  - Fermented tree bark (Shandylia, 1983).
  - Composted pine bark (Rainey et al., 1987; Shandilya, 1989; Hodgkinson et al., 2002).
  - Composted fine bark (conifer and broadleaf bark) (Noble and Dobrovin-Pennington, 2001).
  - Composted conifer bark (Noble et al., 2003).
  - Composted wattle bark from timber industry (Afewerki and Korsten, 2008; Van Jaarsveld and Korsten, 2008).
  - Actilex, ground and composted bark (D'Hardemare, 1985).
  - Composted sawdust (Clancy and Horton, 1981; Nair and Bradley, 1981).
  - Fiber-mix, by-product of a process involving the extraction of polyphenolic resins from the bark of *Pinus radiata* (Rainey et al., 1987).
  - Wood waste from the debarking operations at a pine pole treatment plant, treated in an explosion digester system (Mamers and Menz, 1981).
  - Defibrated pine wood and bark (Clancy and Horton, 1981; Nair and Bradley, 1981).
- Waste products of the sugar industry:
  - Spent lime. Co-product from the processing of sugar beets (Visscher, 1982, 1988; Dergham, 1992; Huerta et al., 2001; Maas, 2003; Beyer, 2004; Pardo, 2008).
  - Sugarcane bagasse (Clancy and Horton, 1981; Nair and Bradley, 1981; Afewerki and Korsten, 2008; Van Jaarsveld and Korsten, 2008).
  - Sugarcane press mud (Dhar et al., 2006).
  - Filter mud, filter cake (Nair and Bradley, 1981; Afewerki and Korsten, 2008; Van Jaarsveld and Korsten, 2008).
- Sisal waste (Hayes, 1978).
- Ground coconut husk (Hayes, 1978).
- Wheaten chaff (Clancy and Horton, 1981).
- Active charcoal (Stoller 1979a, 1979b; Atkins, 1979; Verbeke and Overstyns, 1991; Fermor et al., 2000; Noble et al., 2003; Dobrovin-Pennington et al., 2008).

- Composted cotton husks (Eicker and van Greuning, 1989).
- Tea production waste, dried straw and fiber of tea leaves after manufacturing process (Gülser and Pekşen, 2003).
- Composted vine shoots (Pardo et al., 2002c; 2003; De Juan et al., 2003).
- Straw washed with hot water (Kurtzman, 2000).
- Spent hops (Hayes et al., 1978).
- Cotton ginning mill waste (Garcha and Sekhon, 1981).
- Spent paddy straw after its use for *Volvariella volvacea* (Garcha and Sekhon, 1981).
- Burned rice husk (Singh and Saini, 1993; Khanna et al., 1995; Angrish et al., 2003; Dhar et al., 2006; Om et al., 2008).
- Coffee grounds recovered from an instant coffee factory (Eicker and van Greuning, 1989).
- Fly ash, high temperature residue formed during the coal combustion process and collected as a waste by-product (Wuest and Beyer, 1996; Beyer, 2004).
- Composted azolla (azo-compost) (Riahi and Zamani, 2008).
- Cellulosic materials related to paper and paper industry:
  - Crumbled/shredded waste paper (Dergham et al., 1991; Dergham, 1992; Lelley et al., 1994; Dergham and Lelley, 1995; Sassine et al., 2005).
  - Composted waste paper (Sassine et al., 2005).
  - Shredded newspaper (Stoller, 1979a; Atkins, 1979).
  - Paper and pulp-mill by-product (PPMB). Residual lignin and cellulose fibers from the mechanical and chemical treatment of wood in the preparation of pulp for the manufacture of paper (Hayes et al., 1978; Yeo and Hayes, 1979; Hayes, 1981).
  - Filter paper (Bels-Koning, 1950).
  - Paper mulch (Garcha and Sekhon, 1981).
  - Paper pulp leached for at least 2 years (Eicker and van Greuning, 1989).
  - Paper sludge waste (Lelley, 2000; Cai et al., 2002). Waste paper sludge from newsprint manufacture (Noble and Dobrovin-Pennington, 2001).
  - Champyros®. A mixture of crumbled and composted used paper, peat, and calcium carbonate. pH 7.8–8.1, pore space 85.5–87.0, water retaining capacity 336–344 (Lelley et al., 1994).
  - RPC+®. Recycled newspaper, fiberized and formed into roughened pellets (Morris and Wuest, 1995; Wuest and Beyer, 1996).
  - Graypete®. Synthetic casing which is based on recycled cellulosic wastes such as paper and cardboard to which are added a number of ingredients which improve the properties of the raw materials (Clancy and Horton, 1981).
- Byproducts from the mining industry:
  - MRF (Dewhurst, 2003).
  - Coal tailings, by-product from the washing process for coal to be used in burning for energy (Noble and Dobrovin-Pennington, 2004; Beyer, 2004).
  - Mine-dump tailings (Van Jaarsveld and Korsten, 2008).

7.2.3 Synthetic Materials

- Hygromull®, urea formaldehyde foam (Visscher, 1979, 1982).
- Styromull®, expanded polystyrene (Visscher, 1979, 1982).
- Polyurethane (Barnard, 1974; Visscher, 1979).
- Waterstore®, polyacrylamide (Visscher, 1988).
- Hydratex®, polyacrylamide gel (Castle, 1993).
- Non-ionic gels (agarose, acryl-amide-starch, methylcellulose) (Kurtzman, 1996a, 1996b, 1997, 1999).

- Algin®, natural cationic gel (Kurtzman, 1996a, 1999).
- Dowex®, ion exchange resins (Kurtzman, 1996a, 1997, 1999).
- Stockosorb®. A cross-linked polyacrylamide, polymer material that has the ability to absorb many times its weight in water (Dergham 2000; Beyer et al., 2002).
- Chemical soil conditioners. HPAN (hydrolyzed polyacrylanitrite) and VAMA (copolymer of vinyl acetate and maleic anhydride) (Reeve et al., 1960).
- Woven glass fiber (Nair and Hayes, 1974, 1975).
- Silica gel (Noble et al., 2003).

7.2.4 Other Materials

In this group, different materials that, by their nature or origin, cannot be fitted in any of the previous items are included.

- Spent mushroom substrate (Sinden, 1971; Wuest, 1974, 1976; Allen, 1976; Nair, 1976, 1977; Hayes and Shandilya, 1977; Happ and Wuest, 1979; Stoller, 1979b; Brosius, 1981; Nair and Bradley, 1981; Shandilya and Agarwala, 1983; Eicker and van Greuning, 1989; Shandilya, 1989; Szmidt, 1994; Khanna et al., 1995; Riahi et al., 1998; Rinker, 2002; Angrish et al., 2003; Dhar et al., 2003, 2006; Beyer, 2004; Riahi and Arab, 2004; Barry et al., 2008; Choudhary et al., 2009; Pardo and Pardo, 2008; Pardo-Giménez et al., 2010, 2011, 2012).
- Composted sawdust after bottle culture of *Pleurotus ostreatus* (Kim et al., 1998).
- Spawn run compost (CAC) (MacCanna, 1972; MacCanna and Flanaghan, 1972; Nair and Hayes, 1974, 1975; Dawson, 1978; MacCanna, 1983; Samp, 1993; Tschierpe, 1999; Zied et al., 2010).
- Commercial casing inoculum (CCI) (Green, 1990; Markowitz, 1991; Janssen, 1993; Samp, 1993; Romaine and Schlagnhaufer, 1993; Miller et al., 1995; Bodine, 2005).
- Manure spawn (Allen, 1976; MacCanna, 1983).
- Recycled casing (MacCanna, 1972; Flegg, 1975; Nair and Bradley, 1981; Tschierpe, 1982; Nair, 1983, 1985; Jablonsky and Srb, 1989; Farsi et al., 2011; Oei, 2011; Pecchia and Beyer, 2013).
- Cowdung (farm yard manure, FYM) (Hayes and Shandilya, 1977; Shandilya, 1978, 1989; Shandilya and Agarwala, 1983; Saini and Prashar, 1992; Singh and Saini, 1993; Khanna et al., 1995; Angrish et al., 2003; Dhar et al., 2003, 2006; Suman and Paliyal, 2004; Choudhary et al., 2009; Chandra et al., 2014).
- Cabutz, solid fraction of the digested slurry from the cattle manure thermophilic methane fermentation process (Levanon et al., 1984, 1986; Danai and Levanon, 1996).
- Starch (Kurtzman, 1999).
- Starch-based water absorbents (Stamets, 2000).
- Animal charcoal (Noble et al., 2003).
- Vermicompost (Di Fiore and Albarracín, 1998; Dhar et al., 2003; Choudhary et al., 2009; Choudhary, 2011; Chandra et al., 2014).
  - Farmyard manure based vermicompost (Tomati et al., 1989; Jarial and Shandilya, 2005a).
  - Vermicompost prepared by using spent mushroom substrate from *Pleurotus ostreatus* cultivation (García et al., 2005).
  - Castings of earthworms fed with spent composts and sawdust (Shieh and Wang, 1981).
  - Municipal waste based vermicompost (Jarial and Shandilya, 2005).
- Municipal waste compost (Lelley et al., 1979; Visscher, 1988; Dhar et al., 2003, 2006).
- Composted mushroom stalks (Eicker and van Greuning, 1989).
- Orfa coarse fiber. Domestic waste is ground, metals and glass are removed, organic matter is treated with ozone for odor control, and the product is dried and baled (Wuest and Muthersbaugh, 1990; Wuest and Beyer, 1996).
- Digested biogas plant slurry (Khanna et al., 1995; Angrish et al., 2003).

7.3 Casing Related Techniques

7.3.1 Reuse of Casing

The limitations of the use of peats, mainly by availability and price, have led to the study, in addition to the use of alternative materials, of the possibility to reuse the casing layer in new production cycles. As a general rule, the regeneration process of casing material involves recovering it from mushroom beds at the end of cropping, leaching it to remove soluble salts, and pasteurizing it to kill disease organisms (Nair, 1985). Another advantage of the separation of casing is the availability of soil-free spent compost for alternative uses, with higher value for fertilizing purposes.

MacCanna and Flanagan (1972) reused a sterilized casing from a previous crop cased with a peat and ground limestone mixture. Although sterilized reused casing material tended to reduce yields, they suggest that the use of such material would give reasonable yields in the event of a temporary scarcity of peat. In the work of Flegg (1975), a peat-lump chalk casing was scraped off after cook-out and reused to case a new crop. It caused a slight delay in the start of cropping, and an overall loss of crop of about 11%. According to this author, washing the casing before the reuse or mixing with fresh casing may improve this performance by lowering the concentration of soluble salts accumulated during the preceding crop.

In Australia, Nair and Bradley (1981) used spent casing peat leached in tap water, mixed with peat moss (1:1, v/v) and pasteurized. This spent casing has very good potential for being developed as casing material to replace imported materials, taking into account that yields were similar to that obtained with peat moss based casing used as a control. Shortly afterwards, Nair (1983, 1985) adopted a technique called *counter current extraction*, originally designed for extraction of sliced sugar beet, for the regeneration of spent peat. Material was collected from a commercial mushroom farm by mechanical means using an automatic commercial machine line. After regeneration, spent peat, leached, and pasteurized, was mixed with fresh peat moss and applied to mushroom beds in a commercial farm. Several tests were carried out and yield was at least as good as that from casing control. The author suggests that the counter current extractor process for regenerating spent peat may find an application in commercial cultivation, with consequent reduction in the cost of mushroom production.

At the same time, in Switzerland, Tschierpe (1982) proposed the reuse of old casing material after one year, though an effective pasteurization or treatment with chemicals is essential, and sufficient land should be available for weathering purposes.

Jablonsky and Srb (1989) studied the repeated use of casing soil. The results obtained point to the possibility of the repeated use of soil after steam treatment (60°C, 12 h) without a reduction in yield being recorded going at least three cycles without the need for washing of the casing soil used. Problems associated with the repeated use of casing soil are a gradually increasing number of pathogens, the salinity of the casing soil, changes in water capacity, the structure of the soil, pH, and the problem of removal of the casing soil from the compost. For practical application in the shelf system, the casing soil intended for repeated use can be placed on the surface of the colonized compost on a nylon net. When the cultures are being removed then first of all the casing soil is separated off on its own net and then the layer of spent compost on its own is removed from shelves on a further net. The method of separating casing from compost with a plastic mesh had been previously described by Hesling (1981) without interference with the mycelial growth into the casing. More recently, Farsi et al. (2011) evaluated the reuse of peat-based casing soil by using a plastic mesh in the block system. After harvesting three flushes, plastic mesh with the used casing was lifted from the blocks. Material was piled for three weeks, leached with distilled water and pasteurized (60°C, 6 h). Finally, recycled

casing was applied in a second crop. There were not significant differences between yield of mushrooms from blocks cased with used casing soil and that obtained from original casing material used as control.

In the Netherlands, an installation specifically designed to separate the casing soil from the spent mushroom compost has been recently developed (Oei, 2011). Cultivation trials run using mixtures with the reused casing soil after grinding and treating it in a steam heated auger (70°C) gave good yields of mushrooms when high concentrations were used (50–75% reused), almost matching those of beds using 100% fresh casing soil (Oei, 2011).

Finally, Pecchia and Beyer (2013) conducted experiments to test the impact of different quantities of recycled casing mixed with fresh peat on crop yield. Mushroom yields tend to decrease with an increased rate of recycled casing incorporated into the casing. Taking into account that there was a negative correlation between soluble salt concentrations and mushroom yield, it appears that conductivity plays a key role in limiting the amount of recycling casing material that can be mixed with fresh peat to produce a new crop. A similar situation occurs with the use of spent mushroom substrate as casing (Pardo-Giménez et al., 2012). Methods of inexpensive and effective reduction of the soluble salts content must be investigated for the successful use of recycled casing.

7.3.2 Ruffling

With dense materials that are compacted during the vegetative phase, it is necessary to generate an open texture in order to achieve uniform and abundant fructification and to prevent the production of too much carbon dioxide during fructification and harvesting. This fact led to the introduction, in the 1970's, of deep "scratching" or "ruffling." This process consists of mixing all the casing layer with the mushroom mycelium, which is growing in it, about a week after casing. The result of this procedure is a more open structure which facilitates the interchange of CO_2 and O_2 (van Gils, 1988) and ensures that all the mycelium appearing at the surface is at the same development stage, giving a much more even spread of the primordia over the entire bed surface (Vedder, 1989).

The background of this technique is found in the work of Flegg (1967). Different experiments were carried out to study the effect of a vigorous disturbance of casing layer. When the casing layer was almost completely colonized, it was either left undisturbed or emptied out, broken up, and replaced.

The ruffling gives rise to "mountains and valleys," in such a way that the pinheads are preferably formed in the valleys where they will suffer less from the air-streams, especially when they are young and vulnerable (Visscher, 1975). Dawson (1978) observed a more uniform distribution of mushrooms, although there was no significant increase in yield. However, Visscher (1988) obtained a greater stimulation of fruiting and higher yields with casings of compact texture during the vegetative phase and loose during the generative phase.

This technique is particularly effective with strains of difficult fruiting, where the mycelium has a tendency to continue their vegetative development (D'Hardemare and Mazuel, 1986).

Experience shows that good ruffling allows more accurate control of the first picking day (Buth, 2006). Ruffling is an aid for the diffusion of CO_2 and the admission of O_2 when the growing room is aerated, and it also contributes to a more rapid and uniform colonization by the mushroom mycelium, so that a more uniform fruiting in size and time is achieved. The first consequence is that the mycelium is fragmented, the content of a number of cells comes out, and the action of the bacteria in the hyphosphere is stimulated (Visscher, 1988).

In addition to the improvement of the structure, if the casing layer is not the same depth all over the beds, ruffling can aid to even out irregularities in mycelial growth (Tschierpe, 1981;

Figure 7.3 Hand-operated ruffling apparatus.

Flegg, 1989). From the standpoint of hygiene, however, this method can be dangerous (Tschierpe, 1981). Another variable factor during ruffling is the way the soil is compacted (Van Gerwen and Hilkens, 2004).

The ruffling operation can be carried out manually by means of a flat board with nails worked with a back and forth or circular motion over the beds (Visscher, 1988; Flegg, 1989), or with the aid of a hand-operated rod with teeth (Figure 7.3), but also mechanically with fully electric or hydraulically driven machines specifically developed for use with the shelf system of growing (Figure 7.4). In this case, the intensity of ruffling depends on the number of times the machinery passes over the beds, how deeply the pins or teeth penetrate the casing soil, the number of teeth, their size and shape, the configuration of the teeth on the rod, the space around the rod, the speed at which the rod revolves, and how quickly the machine moves over the bed (Van Gerwen and Hilkens, 2004; Buth, 2006). In the case of manual ruffling, the experience of the operators is crucial for a successful operation.

7.3.3 CACing Technique

The CACing technique ("Compost Added at Casing") involves the application of small amounts of compost fully colonized (spawn-run compost) by the mushroom mycelium to the casing layer at casing time. The technique was developed in Ireland during the 1960s and reported for the first time by MacCanna and Flanagan (1972).

This addition has the effect of assisting a rapid and even growth of mycelium through the casing layer (MacCanna, 1983). The CACing technique has several advantages, and the most important are: time saving (earlier first flush by 3–4 d), more uniform distribution of mushrooms over the entire bed surface, elimination of soil-breaking pins resulting in cleaner mushrooms, no requirement for leveling, no need for ruffling, and a reduction in clumping (Ganney and Stanley-Evans, 1973; Vedder, 1989; Samp, 1993).

In the CACing technique, it is vital that the compost mixed into the casing layer is well run and free of possible diseases and pests commonly found in compost (such as weed molds, bacteria, virus, mites, and nematodes). The obvious advantages of using this technique can be

Figure 7.4 Detail of a ruffling machine: rod with teeth and pressure roller.

nullified by hygiene problems: if contaminated compost is used, the contamination will be spread to the areas inoculated with it (Ganney and Stanley-Evans, 1973; MacCanna, 1983). General precautions to be followed are:

- use the same spawn strain for the casing as for the compost;
- avoid the use of compost that has been overheated;
- remove pieces with black spots or that are excessively dry;
- ensure exclusion of pests and diseases and discard material if it is positive for contaminations such as the presence of nematodes; and
- pay special attention to the hygiene conditions of facilities and equipment.

In addition, an even distribution and proper mixing of material on the casing is vital for the success of the CACing technique (Tschierpe, 1999). In practice, amounts between 0.125 and 2 kg m^{-2} of slightly crushed spawn-run compost are used in growing areas (MacCanna, 1983; Vedder, 1989; Tschierpe, 1999). The amount of CAC influences the structure of the casing soil, the pore volume, the gas exchange capacity and the amount of mycelium (Hilkens, 2012).

7.3.4 Commercial Casing Inoculums

As an alternative to CACing, commercial products are specifically inoculated for use in the casing to eliminate this risk factor of potential spreading of contamination, although it is more expensive (Green, 1990; Miller et al., 1995). A system of using manure spawn instead CACing was previously suggested by Ganney and Stanley-Evans (1973).

Spawn makers have developed different casing inoculums (CCI) prepared on sterile substrates, ususally granulate, in a form suitable for easy distribution in the casing soil at time of mixing, providing the nutrition required by the mycelium, with the opportunity of gaining all the advantages of CACing with none of the risks (Romanens et al., 1989; Green, 1990; Markowitz, 1991; Samp, 1993; Miller et al., 1995; Bodine, 2005). Thus, growers have a safe, very convenient, and relatively low-cost method to use spawned casing (Green, 1990).

The main advantages of these commercial inoculums are: reducing the risk of contamination, shortening of the growing cycle, no need for ruffling, best quality, and ease of harvesting, without clumps and without appearance of mushrooms in deep layers, and the possibility of immediate casing. Shortening the cycle also reduces the generation of flies and thus the incidence of the diseases they transmit, such as dry bubble.

Regarding the doses used, the rate of use per surface area has steadily declined over the years, up to around 60 g m^{-2} (Green, 2004).

Examples of such products are PAC Casing Spawn (Amycel), CMS CACing Spawn (Hollanderspawn B.V.), Lambert Spawn's Casing Inoculum (Lambert Spawn Co.), and Sylvan CI (Sylvan Inc.).

7.3.5 Other Techniques

7.3.5.1 Supplementary Casing Material Addition ("Patching")

The thickness of the casing layer should be as uniform as possible. According to Stamets and Chilton (1983a), an uneven casing depth is undesirable for two reasons: risk of overwatering in shallower regions and irregular pinhead formation. The operation commonly known as "patching" consists of the addition of casing material on the shallow areas, where the mycelium appears prematurely. Thus, an even mycelial spread is assured (Stamets and Chilton, 1983b).

7.3.5.2 Compacting

Compacting the soil after casing and/or after ruffling is a growing technique that can be used to regulate the fructification and, accordingly, the yield and size of mushrooms. This technique modifies the density of the casing layer, mainly affecting the porosity and the air-water relationship. The effect of compaction is not always the same and depends, among other factors, on the size of air spaces, the materials used, and its moisture content. There is always the chance that the casing soil is compacted too much, with insufficient air filled porosity and poor respiration, obstructing the passage for mycelium. This upsets pinheading and the yield remains low. Furthermore, the negative effect of compaction is increased if the soil is dry (van Gils, 1988).

The work of Den Ouden (2005) describes the use of a leveling axle or rod to correct small differences in the surface of the casing layer after ruffling or CACing, and a pressure roller to free the casing soil surface of any remaining lumps of soil.

7.3.5.3 Double Cropping: Casing Substitution

The weakening of the mycelium in the compost and the deterioration of the sanitary status of casing with the progress of the growing cycle, led Talon and d'Hardemare (1979) to develop a technique to remove the casing layer after the second flush, proceeding to fill back the compost into trays, at the rate of three times the original weight per square meter, and to case it with new fresh casing.

Despite the reduction in cropping area, crop quality was improved and compost productivity was maintained with some white type strains, by reducing the incidence of dry bubble and bacterial blotch. However, "weeping" mushrooms tend to appear with susceptible cream and white strains. In addition, operational difficulties and labor costs constitute another important limitation.

7.3.5.4 Double Cropping: Re-Supplementing and Re-Casing

Removal of the casing layer, re-supplementing spent compost after one or two breaks and re-casing offers an opportunity to growers to obtain additional mushroom production from the same compost. This technique can increase yields by 40% or more (Royse, 2008; Royse et al.,

2008). For commercial application, different aspects should be taken into account, mainly the costs of materials and labor, duration of the cropping cycle, sanitary status, and contamination risk.

7.3.5.5 Plastic Film Coverage

The use of a macroperforated polyethylene film (6 mm in diameter) on the casing soil during the first week after casing was proposed by Vedie (1990, 1995), in order to improve the synchronization of fruit body development under the variable climatic conditions of mushroom production in caves. The film was maintained on the casing layer for 9 days. After removing the plastic film, casing was ruffled and a fungicide treatment was applied. This technique resulted in an increase of yield, a shorter first flush, a better timing of the beginning of picking and a higher percentage of smaller mushrooms.

Previously, Gushue (1988) described a technique referred to as *split casing* that involves covering the casing layer with polyfilm to delay pinning on part of the house, in order to regulate production.

7.3.5.6 Sandwich Technique

The sandwich technique is a novel method to shorten the mushroom cropping cycle by about one week with no impact on yield. It overlaps the last week of spawn run with the first week of case hold (Spear, 1998). Spawning and CAC rates are not changed, but a small amount of top-dressing, to provide immediate sustenance for the mycelium in the casing layer, is spread on top of the compost after spawning. The use of CCI for top-dressing at rates of 270 g m^{-2} worked well. Obviously, the technique is not applicable if Phase III bulk compost is used.

7.3.5.7 Ditch Technique

This is a physical separation between the two halves of the bed formed by a shallow ditch running along the middle, in order to improve the performance of manual harvesting (Van Gerwen and Hilkens, 2004). The ditch is pressed down harder than the rest of the casing soil using a wheel mounted to the ruffling machine, so produces fewer or no mushrooms in the first flush. This line sets the limit of the area assigned to each picker.

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8

The Bag or Block System of *Agaricus* Mushroom Growing

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8.1 Overview of the System

The bag/block *Agaricus* mushroom growing system is one in which various aspects of the *Agaricus* mushroom cultural cycle are performed in portable, flexible plastic containers. The plastic containers themselves can be somewhat irregular, circular plastic sacks (bags) or formed rectangular blocks of variable size and dimensions, but usually 0.4 m wide × 0.6 m long × 0.15 m deep. Traditionally the bags/blocks are filled with phase II compost, incubation is done in the bag/block, and the bag/blocks are cased either individually or in series. The case run and pinning is then performed and the mushrooms are grown out on the plastic container for an 8-week cycle. Alternatively, the bag/block can be filled with phase-III compost, cased, and then grown out for a shorter 6-week cropping cycle. In some circumstances blocks are used as a vehicle for transport as they are formed with phase II compost, incubated, transported, and then broken open to fill trays, beds, or aluminum shelves as phase-III compost for cropping (Samp, 2002).

From this general description of the bag/block growing system one can see that the system is very flexible and adaptable. Most simply, it is an inexpensive system to enter the mushroom business, it is an easily expandable system, and can be altered in many ways to give whatever the desired result. In my experience with the system I have worked with some of the smallest operations (less than 100 m^2 cased/week) up to very large block mushroom farms that case up to 7000 m^2 per week of blocks. Used correctly, the system is capable of growing equal or better yields (in kg m^{-2} or kg ton^{-1} of compost) of equal quality as any other type of mushroom farm including aluminum shelves. These advantages would seem to make the bag/block system superior to any other because of its flexibility, cost effectiveness, and positive results.

Along with all the inherent positives of the bag/block system of mushroom growing there are some aspects that make it somewhat specific to certain geographical regions. This is in part *because of* the portable, flexible nature of the system. Bags/blocks can be formed to any size and/or filled to any weight, but normally each container weighs between 16 and 25 kg. Bag/block weight can range from 16–25 kg per unit, but most farms operate at ~18–20 kg. Because bags are not well formed at time of mechanical fill they are usually manually lifted, formed, positioned, and cased by hand. This makes bag fill and placement labor intensive, normally suited to men only, and creates a "busy" environment. Because blocks are mechanically compressed to a given size and shape (again 0.4 m wide × 0.6 m long × 0.15–0.2 m deep) the system

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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can be mechanized in various ways. Having said that, mechanization is not as defined as other cultural systems and generally is unique to the site in question.

8.2 Bags and Blocks in Use

Variations of the bag/block system have been used in many mushroom growing countries around the world. The Irish concept of satellite growing started with the bag system in the 1980s. Pat Walsh and his company Custom Composts heralded a system whereby compost was made at a central phase I and phase II composting site, bagged as phase II compost, and distributed to a network of dedicated, family run mushroom farms that grew out the mushrooms. The mushrooms were collected by a marketing agency and then were sold and distributed to markets in the United Kingdom. In this case the composting and sales divisions were owned and managed by Walsh and the large network of satellite farms operated as individual, wholly owned farms provided the product.

Since the farms were owned by individuals they operated with an owner's mentality. Quality was the mantra and the bag system with a single layer of bags on the floor of hooped, polythene covered growing rooms provided the superior quality for the local Irish market, but mainly for export (Figure 8.1). The satellite system, as designed and managed by Walsh, was a great system of integrated and interdependent relationships that turned out the quality and volume that brought the long-established British mushroom industry to its knees. As it was, the exported quality was so good and the efficiency was so superior that the home-based British industry could not compete with respect to either quality or price. Even selling at lower prices than their British competitors, the compost yard, the sales and marketing effort, and the individual growers made handsome profits. Such was the strength of the system and the industry until the turn of the century.

From this blueprint others learned quickly and followed Walsh into the satellite system of bag growing. At its peak in the late 1990s, Ireland had about 12 compost yards shipping out bagged compost to over 1000 growers. As mentioned, the flood of mushrooms from all these growers eventually put most of the English mushroom growers out of business. Whether Walsh was the first or one of the first is not important. What is important is that a flexible, inexpensive means of growing competitive yields of high quality mushrooms was created and that template was followed in satellite ventures in Hungary, Poland, Russia, Australia, Latin America, and other countries. As time passed many of the Irish central compost yards eventually morphed their growers into more efficient and cost effective staged bag farms (multiple levels of bags), then multiple staged *block* farms, and then *phase-III block* farms (blocks with fully colonized compost). These phase-III farms had the benefit of more annual throughput via more blocks per room plus a cropping cycle that was 2 weeks shorter. From these developments one can see that the general trend was from bag growing to block growing. The use of blocks made operations easier, allowed some mechanization, and most importantly increased the number of units (and tons of compost) per room because of their smaller and more regular shape. The evolution of the bag/block system, as with any production system, followed the trend to increased efficiency and cost effectiveness.

Beyond the satellite model, standalone bag/block farms could be found all over the world. In most cases a farm would produce its own phase I and phase II compost, bag or block it, and then manage its own cropping process. These farms tended to be larger because of the added cost and scope that comes with producing and bagging its own compost. Vertically integrating all aspects of the cultural process requires more machinery, space, and management, which in turn requires more throughputs to pay for it. Having said that, I've been associated with

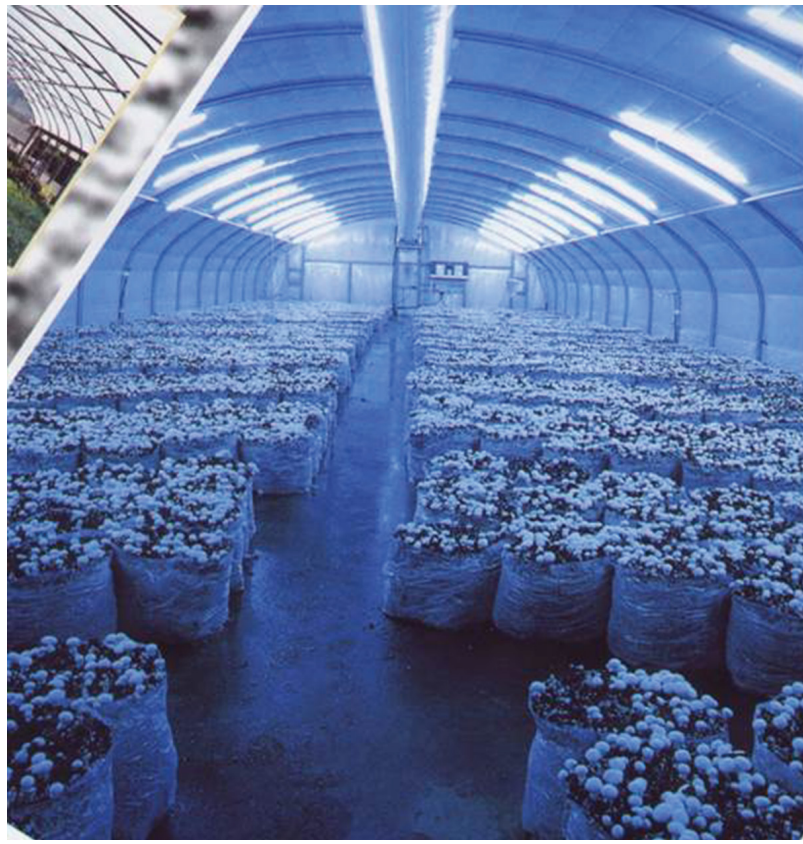

Figure 8.1 Superior quality of bag system with a single layer of bags on the floor.

standalone bag/block operations that produce off as little as 250 m^2 cased per week up to 7000 m^2 cased per week. The magnitude and sophistication of the operation is almost always reflective of the amount of compost produced or area cased.

Finally, the other application of bag/block utilization is as a transport vehicle for phase II or phase-III compost. There are block producers in Holland, Australia, and the United States that transport their product large distances to customers wanting to grow out mushrooms, but do not have compost capabilities. These blocks are produced in the country of origin by a commercial compost producer and sent via container overseas or via truck overland to contracted growers. Once received these blocks are either grown out in the blocks themselves or the blocks are broken open and the compost grown out in trays, beds, or shelves. In some cases, the blocks are shipped in refrigerated containers as phase II compost and arrive as phase-III compost allowing the grower to spawn run in the container and shorten his cropping cycle. In another situation fully grown phase-III blocked compost is trucked cross-continent to be broken open and filled onto shelves. Such is the flexibility of the bag/block option.

As mentioned earlier, most of these standalone bag/block operations are in regions of plentiful or inexpensive labor. At the time of writing this article (2015) most bag or block operations are in Latin America, Africa, and Asia following the tendency of operating in labor abundant locations. Although that is the norm, there are still some block farms in Australia, the United States, and Europe. These tend to be operations that take phase-III blocks, which give the

advantage of a shorter cropping cycle. As mentioned the shorter cropping cycle reduces 2 weeks from the normal 8–10-week cycle and therefore accommodates more room fills per year. It can be said the extra production from greater compost throughput compensates for the extra labor and per unit cost.

8.3 Practical Use of the System – Phase I and Phase II

Phase I and phase II composting can be done in any way that will provide a quality growing medium to be bagged or blocked. The bag/block aspect of the system does not come into play until phase I and phase II composting is finished and the finished phase II compost is filled into the plastic container. As far as the composting process is concerned, the compost can be prepared in bunkers or stacks or any combination of which to arrive at a superior finished product. The best and most efficient arrangement of compost production would be bunker produced phase I compost and tunnel processed phase II compost. In this way, the compost can be produced most efficiently and be the most productive if proper composting principles are adhered to.

Alternatively, bag/block operations exist that produce compost in stacks (ricks) with compost turners and other conventional equipment. The finished compost is then filled into trays for pasteurization and phase II. In this case, the finished phase II compost would be tipped from the trays into a hopper and filled into bags or blocks for incubation. The only compost raw material requirement is that the straws are not excessively rigid or tough since they might perforate the plastic when bags or blocks are compressed, such as with soy or maize stalks.

8.4 Practical Use of the System – Spawning and Phase III

As with any *Agaricus* mushroom growing system, spawning and bag/blocking must be done in a hygienic manner. Once pasteurization and phase II is complete the compost is vulnerable to contamination once it exits the confines of the pasteurization vessel. As such great care must be taken with respect to air movement into the spawning area, cleanliness of equipment, cleanliness of the workers, and the spawning area in general.

Ideally the spawning and bag/blocking area should be isolated and be clinically clean prior to a spawning operation. The bagging/blocking area should be overpressured with filtered fresh air (1 micron or less) and critically cleaned and disinfected prior to opening the phase II doors. No flies should ever be present in the spawning area. Exposure to unfiltered outside air and mushroom house exhaust air is particularly dangerous. Exhaust from rooms in which mushrooms are grown can carry virus carrying mushroom spores as well as other microbes, spores, or flies that may be present during cropping. Once the compost is filled into the plastic container some of the danger is relieved because of the prophylactic nature of the plastic bag. This in itself is an important advantage of the bag/block system because the plastic physically keeps spores, flies, and any other contaminant out of the compost. Even with this advantage, constant attention should be paid to an integrated pest management program to keep pathogens and competitors at nil or at least under strict control.

Aside: I worked with a bag operation for many years that exhibited outstanding production and quality until a *Trichoderma* infection commenced. It turned out to be an aggressive strain of *Trichoderma harzianum* (*aggressivum*). Because it was a relatively small farm (~300 m^2 per week) the harvesting area was adjacent to the spawn and case area which was open to outside air with no overpressure and certainly no filtration. The *Trichoderma* infection could *not* be

cleaned-up after years of low/non-capital expenditure exercises and yields eventually degraded by 25%. In this case ~$10,000 USD could have solved the problem, but it was not done. The illustration is proof perfect that the plastic used to encapsulate the compost does not absolutely protect one from pests and disease.

At the time of spawning the compost is taken from the phase II tunnel or tray phase II room and loaded into a type of metering hopper that levels the compost. This level flow of compost is essential since the compost then flows past a constant metered flow of spawn, and supplement if added. The constant flow of equal volumes of compost, spawn, and supplement allows them to be mixed together in the proper proportions. After proper metering the spawn and supplement is integrated into the compost with mixers prior to bag/block fill. It is imperative that the correct amount of spawn and supplement is thoroughly, evenly, and completely mixed into a given amount of compost. This is critical to all following aspects of the cropping cycle. Poorly applied and/or mixed spawn or supplement will present unnecessary problems in phase-III, pinning, and cropping so it is imperative to get this aspect of the operation correct. The blended compost/spawn/supplement is the foundation for the rest of the cropping cycle. A poor foundation ends in a poor result.

The amount of spawn to be mixed into the compost prior to bagging varies from farm to farm. The range can be 8–18 units of spawn/metric ton of phase II compost. Higher rates of at least 12–18 units/metric ton are preferred to achieve a completely colonized spawn run within a normal ~2-week period of incubation. If added, supplement should be added at recommended rates, which are usually up to 1% of the compost wet weight or up to 3% of the dry weight. Check with your spawn and supplement suppliers for recommended rates of application.

The spawned, and perhaps supplemented, compost then flows from the hopper and spawn/supplement belt into the bagging or blocking machine. The bagging machine is usually a carousel design that rotates a large, 5/6-holed disk with an unfilled bag attached under each hole (Figure 8.2).

The spawned/supplemented compost is delivered to the carousel by a fixed belt and flows through the disk-hole into the bag. The bag is filled with a weight actuating or timed trigger that advances the disk when desired bag weight is achieved. The filled bag is then manually removed from the disk and an empty one put in its place. The filled bag surface area is around 0.2 m^2 and the weight per bag varies from around 18–25 kg. The objective must be to fill each bag with the same amount of compost, the same number of spawn particles, and the same amount of supplement. Finally, when the bag is removed from the carousel it is palletized so it may be delivered to the grower for incubation or the incubation/growing room in a vertically integrated farm (Figure 8.3).

In the case of a blocking operation the same hygiene principles apply. Upon removal from the phase II vessel, the compost is also placed into a metering hopper so that compost, spawn, and supplement flow is controlled to achieve desired ratios. On a typical blocking machine the loose compost flows into a series of individual defined chambers, which serve as the dimensions of the blocks (Figure 8.4). The compost is then pressed into the chamber, wrapped in plastic, sealed, and moved on to palletizing. Once again, block size is usually 0.4 m wide × 0.6 m long × 0.15 m deep and can weigh from 16 to 25 kg. Many blocking machines have flexible compost chamber dimensions so that blocks can be of variable sizes or dimensions. This is very important if one desires to use heavy blocks (>20 kg).

This brings us to the nature of the actual bags/blocks. Bags are generally ~910 mm tall and ~ 410 flat widths equating to surface area of ~ 0.2 m^2/bag. In this case, five bags make up 1 m^3. They are made of 60 μm gage plastic of which there are no holes in the bottom half. Some operations have ~5 mm holes in the top half, but some have no holes at all. The purpose of the holes is to allow some gaseous exchange, but as long as there is enough aeration through the

Figure 8.2 Bagging machine.

top of the bag it seems the holes are not absolutely necessary. In fact, holes in the bottom half of the bags are a disadvantage since applied water may percolate to the bottom of the bag and then drop onto the bags below. This can cause staining to the mushrooms of the bags below. Since there are no holes in the bottom of the bag it is not uncommon to see ~5 cm of water in the bottom of bags later in cropping due to leaching of applied waters (and condensation). I have not found it to be a problem.

Plastic used for blocks is bolted plastic of a similar gage that is mechanically wrapped around the formed compost block and then heat shrunk and sealed. Because blocks are completely sealed it is essential that holes are present in the top half of the blocks. Failure to do so will create anaerobic conditions within the block, or at least very high CO_2 concentrations that will slow or even kill mycelial development. As such, bolts of plastic can be drilled with holes or holes are made into the blocks after wrapping. Again, holes are not present in the bottom of the blocks to avoid leakage of leachate onto the blocks below.

Aside: The amount of compost at bag or block fill is a decision made at the management level. This is one of the fundamental decisions made on any mushroom farm and a bag/block farm is no different. There are several considerations regarding weight of the container, some of them cultural, some physical, and some have safety considerations. Culturally as much weight as the bag/block machine and HVAC systems can comfortably handle is the preferred direction. More weight per container gives additional production and quality *potential*. If the high energy derived from the heavy fill of compost can be controlled throughout the process, the energy can be channeled in the desired manner during cropping. That is, the high volume of compost may be directed in a quantity preferred or quality preferred manner. Physically, space is a consideration regarding bag/block weight. There must always be enough space for watering and harvesting so the size, and therefore the weight, may be limited by the space available. Also, a slight advantage of production efficiency goes toward lower weight bags/blocks, but in my opinion that small advantage is of secondary importance. Finally, the safety aspect of bag/block

Figure 8.3 Spawned blocks (palletizing).

fill is what ultimately limits weight per unit. At some point in all bag/block operations the containers must be manually lifted and moved. In some countries, this can become a worker safety problem and weights are "capped" at around 18–20 kg. In other situations, 25 kg per unit is possible.

One of the great advantages of bags/blocks is compartmentalization. That is, each bag/block is its own entity. As mentioned earlier the compost within the bag or block is somewhat protected by the plastic wrapping from contamination. Furthermore, if any disease or pest is identified later in the cropping cycle, the bag or block may be removed and discarded at any time to eliminate the problem or to limit contamination to other bags/blocks. Another benefit of compartmentalization is that bags/blocks are very forgiving when it comes to release of metabolic heat from spawn run or cropping. When excessive heat *does* occur within a bag or block in many cases only the very center of the container is adversely affected leaving viable mycelium all around the plastic surface. In some cases, the heat compromised compost may be recolonized later in the process to give at least some benefit. Other systems only recover minimally, if at all. The reason for this benefit is that the surface area to volume ratio is very advantageous to heat transfer out of the bag or block. Additionally, the plastic bags/blocks may be placed on rods, a screen, or perforated base that allows better heat transfer through the bottom of the container than wood (as in trays or beds) or nets plus aluminum (as in shelves). Also without sideboards or aluminum side rails better heat transfer occurs though the sides of the bags. I've seen some cases where operations have been successful with slightly less than optimum cooling capabilities because of the benefit of compartmentalization. However, it must be said that optimal environmental control is always an advantage in mushroom growing to allow for proper temperature control or control of relative humidity.

Another advantage of compartmentalization is that the plastic used for bags/blocks is usually clear. This allows visual access to what is happening inside the bag. One can evaluate

Figure 8.4 Example of blocking machine platform, each separation refers to the block size.

growth of mycelium, need for water, energy release, and the presence of disease. The ability to recognize *Trichoderma*, *Chaetomium*, or even the presence of black or partial black bags just by looking is a great advantage. Also, the plastic wrap is an advantage regarding compost moisture loss. In the case of bags, the bag size is usually ~250–300 taller than the compost in the bag so when the unit is placed for incubation the top can be folded over to conserve moisture (Figure 8.5). This also allows the application of insecticides at the top of the closed bags, thus preventing the entry of flies into the bags. With blocks the compost is completely enclosed with the exception of the holes for gaseous exchange. Because of this there is little moisture loss during spawn run. In the cases of both bags and blocks condensation is normally found inside the bag during the spawn run heat surge to the extent of puddling. In other systems, this moisture is lost, but the plastic containment traps the essential compost moisture inside the bag/block.

Once the bags/blocks have been formed they must be delivered to the incubation or phase-III chamber. This chamber may also be the room in which the mushrooms are grown out or alternatively it can be a dedicated room with higher cooling capabilities. In the former case, the bags/blocks are in position to be cased so at the time of casing the casing soil is brought to the containers. If blocks are placed on standard aluminum shelving the spacing is standard and casing can be mechanized and performed as would a normal shelf farm (Figure 8.6).

Figure 8.5 Bags closed (not hermetically) for the spawn run.

In arrangements where bags/blocks are incubated in the growing room the casing is applied by hand. This can be laborious because the making of the casing, its transfer, and then its application occupies different areas and can become a bit messy. However, because the mushrooms will be harvested in the same position in which the bags/blocks were placed at spawning it eliminates another transfer of containers. Additionally, where bags/blocks are placed in situ for

Figure 8.6 Mechanical casing of blocks winched onto shelves.

harvesting there is more space around the containers allowing for more heat transfer during incubation (Figure 8.7).

In the case of a dedicated spawn running/incubation room the bags/blocks are usually placed in close proximity to get more units in the room. That room can then be fitted with larger cooling systems to remove phase-III metabolic heat rather than fitting all rooms with larger cooling systems. Then after incubation the bags/blocks must be transferred out of the room for casing. Actual bag/block placement in a dedicated spawn room is important and will be discussed in the next paragraph. Some of the benefit of superior heat transfer out of the containers is lost if more than two blocks are placed touching each other. In this case, metabolic heat accumulates without the benefit of easy removal. The best situation is for the bags or blocks to be air positioned so there is air completely around each unit. By positioning the containers in this way, the air flow of the room can remove the heat most efficiently (Figure 8.8a). Having said this, most dedicated incubation rooms allow for some contact between bags or blocks. Once spawn run is complete the bags/blocks are removed from the room and either are brought to an area where they are cased and then transferred to a growing room or brought to the growing room and cased there. Because of the removal to a remote area some sort of casing line and/or mechanization is possible with blocks.

Actual stacking the bags or blocks is almost unique to each operation. Much depends on the size and shape of the rooms. As mentioned earlier, in a dedicated spawn run situation the containers would be placed very close to each other to maximize units per room. Since the containers would never be harvested in the incubation rooms they may be stacked very high (up to eight levels has been seen) and close together, again to get maximum units in the room (Figure 8.8b).

Figure 8.7 Dedicated incubation room with tight bag/block placement, but less than optimum heat transfer.

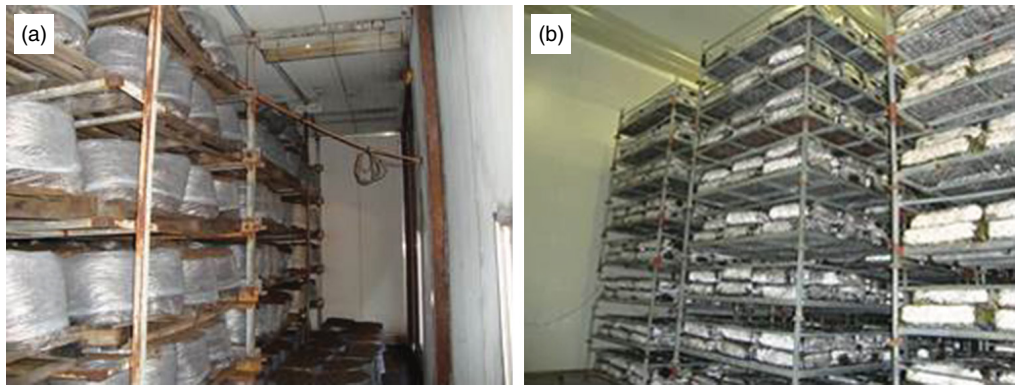

Figure 8.8 Dedicated incubation room for bags (a) and blocks (b).

In each case the blocks would be air positioned on racks with multiple stages (levels). The racks would be stacked the width, length, and height of the room with space for personnel passage and air movement. In sophisticated operations, the racks are of conventional shelves with the blocks placed inside. In this case the filling of the shelves can be mechanized with a standard net and winch with the blocks placed on the net as it is being drawn into the shelf (Figure 8.9). In other cases, racks can be of metal frames with various number of stages (levels) with wire bottoms for maximum heat transfer out of the bottom of the bag/block. In this case the bags/blocks are placed on the stages by hand. On other farms the racks may be composed of metal trays, wood planks, heavy wire mesh, or even wood pallets and bamboo shelves.

Regardless of the method of stacking, optimum air distribution and climate control is imperative. Conditioned air must be delivered to each bag/block similarly to remove metabolic heat

Figure 8.9 Mechanical filling of the shelves.

and allow mycelial colonization of the compost under optimum climatic conditions. Care must be taken to design the room and the air handling systems to work optimally considering the placement of the racks, the number of stages, and the placement of the bags/blocks on the stages. The distribution of conditioned air must be equal vertically, lengthwise, and widthwise within the room. Failure to do so will result in "dead pockets" where containers will heat up excessively or "cold pockets" with excess airflow in which containers will colonize slowly. In most cases contracting an HVAC professional is beneficial to size the environmental control systems correctly and to deliver the air optimally.

8.5 Practical Use of the System – Casing through Cropping

Casing can be the most challenging aspect of the bag/block system because it can be labor intensive. Because of the many small, individual compost containers in many cases they must be cased individually by hand. The method is usually unique to the operation. This is particularly true of bag growing, whereas it is possible to fabricate a casing machine for certain arrangements of block growing (Figure 8.10).

Also, the stacking arrangement of the farm is generally unique to the operation. If blocks are filled onto shelves the shelves are arranged lengthwise (longitudinally) and can be up to six high in standard shelf farm arrangement. Otherwise blocks can be set on individual "trays" that may be stacked to a suitable height (Figure 8.11). This arrangement is suitable for mechanical casing. Alternatively, bags/blocks may be placed on stationary home-made racks to a height and width desired by the farm. In the case of blocks, they can be placed in any arrangement either at spawning or prior to casing to give the optimum access by the harvesters. Because of the 0.4 × 0.6 m block dimensions they can be placed two long or three wide to present a 1.2 meter wide growing area, although other arrangements have been noted. The number of stages varies from farm to farm and may be 2–6 high. Bags on the other hand have fewer stages because of the increased height of bags versus blocks. In the early days of Irish bag growing the bags were stacked on the floor for easy access, although eventually the bags became staged onto two or a maximum of three levels.

Figure 8.10 Casing machine.

Figure 8.11 Individual "trays" with blocks.

The casing itself is of standard mix as any other type of *Agaricus* mushroom growing operation. The main component of the casing is peat moss which may be composed of blond peat (young sphagnum), black peat (old sphagnum), or a blend of the two. The blond peat is usually more economical, but the black peat has physical characteristics that make it preferable to some growers in some markets. In general, the blond peat accepts, but loses water more easily, therefore it requires more applied water (Samp, 1993). The black peat retains water more aggressively so fewer days of water application is possible. Additionally, black peat tends to roll off the mushroom caps if they form under the surface resulting in cleaner mushrooms. As such many times a small percentage of black peat is blended (10–33% by volume) with blond peat to obtain a combination of less expensive casing with some of the characteristics of black peat.

The other component of the casing is limestone, sugar beet lime, or hydrated lime as used in Pennsylvania, US. The purpose of the limestone is to buffer the pH of the acidic peat moss (pH ~3.5–4) to a neutral or slightly alkaline level (~7.2–7.5). In the case of hydrated lime the pH is raised to over 8 before it gradually declines into a neutral range. Other than pH buffering capabilities the lime portion of the casing mix can be used to amend the structure of the casing. Generally speaking, the particle size of the lime influences casing structure. Large particle size of ~3–6 mm gives a more open, porous structure to the casing. Smaller particles to flour consistency results in a denser, heavier casing (Samp, 1995). The use of sugar beet lime amplifies the effects of flour limestone adding a pasty, greasy feel to the casing. This is considered a positive factor since it lends some of the positive characteristics of black peat to a blond peat casing soil.

The casing itself is usually made in a batch system. There are dedicated casing mixers manufactured by various mushroom industry equipment suppliers, otherwise agricultural feed

blenders or even cement mixers can be used. In a batch casing mix system, a given number of bales, the desired amount of limestone product, and the desired amount of water is added to the casing mixer. Ideally a tank or flow meter is associated with the mixer so a constant amount of water is added to each mix to achieve consistent casing moisture from batch to batch. This is very important since varying moisture can affect the density and structure of the casing, which in turn significantly affects the mycelial colonization of the casing. Again, one must do the best they can to ensure batch to batch moisture consistency, but in reality, variable bale to bale peat moss moisture ensures there always is some moisture variability from batch to batch. Because of this some judgment and subjective evaluation must occur at the release of each batch. Finally, the last ingredient added is the CAC (compost added to the casing) material, which is used to colonize the casing rapidly and shorten the time to first flush. Whether proprietary CAC or managed compost is used, it is best added at the last possible moment to get sufficient distribution in the casing without rubbing the mycelium off.

When there is a dedicated incubation/spawn run room the bags/blocks are brought to the casing area (in most cases also the hygienic spawning area) where they are cased and then transferred to the grow out room. Immediately prior to casing the surface of the compost is exposed for direct application. In the case of bags the excess plastic covering the surface of the compost is rolled down the side of the bag to facilitate application of the casing. In the case of blocks the surface plastic (the side with aeration holes) is cut away and removed to expose the compost for casing (Figure 8.12).

Since mechanized casing applicators are usually self-designed and unique to the operation, I'll not go into any details regarding their design or operation. Suffice it to say the casing machine is static and the blocks (or series of blocks on a rack that can be stacked in the growing room) pass under a cascade of leveled casing soil as would occur on a tray farm (Figure 8.13). Beyond that you can use your imagination.

Figure 8.12 Blocks with plastic cut away for casing.

Figure 8.13 Mechanized casing.

Alternatively, in many cases the casing is brought to the room to be cased and the bags or blocks are cased in situ within the room where they were incubated and will be harvested. After a batch of casing is mixed it is released into a portable hopper or container for use. Once again, prior to casing the excess plastic of the bags is rolled down or the surface plastic of the block is cutaway and removed. In some cases, this action is performed the day prior to casing to allow surface condensate moisture to evaporate prior to casing. Then at the operation the casing is transported to the room to be cased and manually transferred to the bags/blocks. There is a degree of "art" associated with how the casing is applied to the surface of the compost. The casing crew first must be fitted with equal volume containers, each of which holds precisely the amount of casing for each bag/block. Then by carefully dumping the casing onto the compost it can be spread to deliver an equal depth on each container.

The next aspect of casing application cannot be overemphasized since it has a major impact on the quantity and quality produced on the first flush and beyond. Once the casing is applied to the bag/block the material must have the same density, structure, and porosity over the total area of the surface. In many cases when a wet/dense casing is placed on the bag/block the casing keeps the structure it had while in the container – dense and compact. If this is the case the density is too high for good mycelial development in the center of the bag/block. For this reason, the casing must be "worked" with the fingers after it is placed on the compost. This will help achieve the same density and porosity over the total area of each bag/block. Finally, the casing must be spread out evenly over the compost surface to give the proper depth and type of surface desired. This is done manually or with the help of a leveling stick.

Aside: Given there is enough quality compost available in the bag/block, some of the best quality at the largest size mix was achievable by using a heavy, wet casing that limited mycelial growth in the casing. By applying the casing in a way to discourage excessive colonization,

better pinset control can be achieved with the size mix required. With one particular client, we regularly achieved 70–80% of the total production >40 mm diameter of dense, high quality mushrooms. Yields were also consistently very high. Of course, other size mixes can be achieved with other types of casing and cultural practices, but that example confirms what is possible.

Case running, pinning, and pin development are cultural processes that are very similar to any other system of *Agaricus* mushroom production. Environmental control now becomes more important for the setting of pins, and more critically, setting the correct number of pins on the first flush. Modern markets require higher quality and that starts with everything that has been presented to this point plus the casing layer and the pinning process for the first flush. This section will be minimal since these cultural practices will be addressed in other chapters. However, the following characteristics that are particular to bag/block growing will be explored.

Regarding the pinning process, the principle is the same as any other growing system. First flush pinsets are determined by the quantity and quality of the compost, the concentration of mycelium available to set pins, and the environmental conditions used to set the pins. Assuming the compost is of good quality and sufficient in quantity, the casing must be colonized properly to support a good pinset. Although this is one of the aspects of the "art" of mushroom growing, it is difficult to definitively describe. Too much mycelium in the casing is undesirable, as is too little. This is largely determined by the casing mix (structure), quality of application, moisture at casing, and moisture addition during colonization of the casing (case run). Generally denser, wetter, heavier casing will result in less colonization that will result in fewer mushrooms (Figure 8.14), whereas looser, drier, finer casing will result in more mushrooms (Samp, 1998).

Figure 8.14 Heavy casing which limits mycelial colonization (approximately 4 cm).

Then the case run must be managed for proper timing. This is determined by the CAC rate, structure of the casing, watering pattern, and the environment. Normally the environment is managed for good mycelial growth into the casing while keeping the compost temperature in an optimum range (~27 degrees). Applied water is important to keep the casing structure and moisture optimum, and allow for correct timing of pinning. The speed with which the mycelium colonizes the casing determines the date of aeration (pinning) and the timing of the first flush.

Back to pinning and pinset control, the characteristics and management of the casing only account for part of the resulting first flush. The timing and type of environmental shock also influence the first break pinset. A harder pinning procedure with a quicker drop of air/compost temperature and CO_2 concentration (example: 15/16°C air and 1000 ppm CO_2 in 36 h) at the proper time will normally result in more first flush mushrooms. A slower drop of temperatures and CO_2 (example: 18°C and 1500 ppm CO_2 in 72 h) will result in fewer mushrooms. If everything (including harvesting) is managed correctly the resulting pinsets can be similar in production, but generally more mushrooms will produce a higher yield at lower quality. The priority is always made by farm management.

Once again pinning, pin development, and grow out is similar to any other cultural system, which is addressed in other chapters. Bag/block growing can be a bit unique because the heat transfer advantages mentioned earlier can work as a disadvantage during cropping. During growing, one must be vigilant that too much heat is not removed from bags/blocks and stifle the metabolic activity of the compost. This can hurt gaseous exchange, evaporation, and timing of the crop. This is more of a problem when bags/blocks are low in weight (16–20 kg) and various adjustments can be made.

First lower fan speed can be used to maintain compost activity as long as other critical parameters are achieved (room temperature, CO_2, relative humidity). This will extract less heat from the bags/blocks. Also, the bags/blocks can be placed on a wide board or wood planks to insulate the bottom and conserve activity. Finally, more bags/blocks can be stacked closer together to act as a bed or shelf. Although each unit is still compartmentalized the heat of many bags/blocks stacked touching each other can conserve heat and promote activity.

Watering during case run, pinning, and growing can be done as in other mushroom farms. Water can be applied via hand watering with an appropriate nozzle or with a watering "tree" (Figure 8.15). As always, the water must be applied consistently with the same volume to every bag/block at the correct pressure. Hand watering is more laborious but allows individual attention to each bag/block whereas watering with a tree is faster and allows all the stages of the bags/blocks to be watered at the same time. The latter approach is less labor intensive and cleaner, but less specific.

Water can be applied for several purposes such as to add density to the casing, to control case run, to add moisture to the compost, to cool the compost during spawn run or case run, to knock back vegetative mycelium after airing, to apply chemicals for disease or pest control (including blotch), or simply to add moisture to the casing soil. In all cases, complete coverage of all the surface area is essential along with measurement of the amount of applied water per application. Ideally, a dedicated, known volume tank supplies the water applied by hand or tree. In this case the exact amount of water at each application is known and can be recorded. This is important to proper moisture management of the crop.

Harvesting is the final production/quality related process and cannot be overemphasized. All the effort, cost, attention, coordination, and so on of the entire growing process comes down to harvesting. Therefore, harvesting should be given the utmost attention. Certainly, harvesting

Figure 8.15 Equipment used for irrigation of the casing layer, called the "water tree." (*See color plate section for the color representation of this figure.)*

management is difficult because the process is manual and its execution is in the hands of many diverse individuals with various agendas. However, the attention paid to the harvesting function always maximizes any farm's qualitative and quantitative performance regardless of the cultural system used. Again, since harvesting will be dealt with elsewhere I'll defer to that section and focus on the peculiarities of harvesting on bags/blocks.

First of all comes the stacking/staging arrangement. This has been discussed previously in this chapter, but if the farm incubates in situ, stacking/staging must be designed in reverse sequence. That means the spatial conditions required for harvesting dictate how the bags/blocks are arranged after spawning. The reason is because in all cases it is essential for the harvesters to see what they are doing. If they cannot see the mushrooms, they cannot execute any training program. Therefore, much thought must be put into the stacking/staging arrangement to allow for visual and physical access to the mushrooms. Optimally the harvester should be able to look down at the mushrooms and be able to access them without reaching (Figure 8.16). That is, the width of the bags/blocks should not be much more than average arm's length. Since this system is widely adjustable, this is easily arranged with intelligent growing room stacking/staging design.

Secondly the picking platform design is critical. Since there are not sideboards/rails the stacks cannot be climbed as in the United States. As such most farms use moveable trolleys that can be climbed to harvest upper stages and that also accommodate a number of boxes for different mushroom grades. The trolleys should always be designed with the stacking/staging in mind for proper height, width, levels, and so on for the average harvester. Additionally, easy movement of empty and full mushroom containers, trolleys, and people must be considered when designing the ideal arrangement of all these considerations. When blocks are used in a standard shelf arrangement, picking lorries can be utilized.

As mentioned earlier disease control with bags/blocks is an advantage over other growing systems since any disease may be easily treated or removed. As would be done with other systems topical disease should be properly treated and covered. The same is true for bags/blocks, although disease spotting can be easier because the stages of bags/blocks are less wide than other systems. Beyond that a diseased bag can be hygienically removed from the crop by

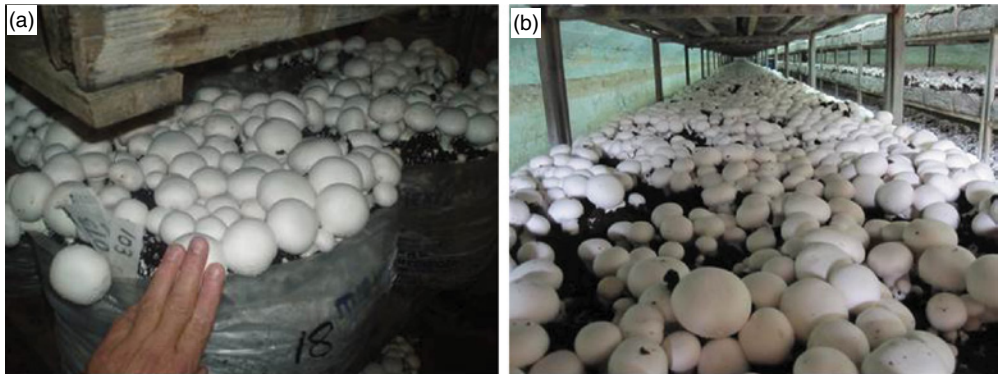

Figure 8.16 Mushrooms accessible for harvest on bags (a) and blocks (b).

rolling back up the top of the bag (it was rolled down at casing), sealing it, and remove it from the room. Blocks cannot be sealed off as bags are, but still can be easily removed from the room.

The last part of the cropping cycle is cook-out or post crop. Crop removal generally is a labor-intensive operation to remove the bags/blocks from the racks for transfer to the removal truck (Figure 8.17). It is always desirable to steam cook-out the crop to kill all viable mycelium and any pests or pathogens before removal from the growing cell. Since disease

Figure 8.17 Individual "trains", an efficient way of disposing of blocks.

control is always a function of controlling load (spores, mycelium, flies) killing of the finished crop ensures the disease load is not compromised by disturbing any established disease with the removal of the bags/blocks. Having said that, many bag/block operations do not cook-out with steam because of the ensuing mess. For sure cooking-out with steam creates many clean out problems because of the elastic nature of plastic. If this is the case the bag/block surface should be sprayed heavily with suitable disinfectant before removing. Bags can be rolled back up and sealed, but not so for blocks. Then complete cleaning and disinfection of the room and environs must occur the same day. No mycelium, mushroom tissue, or compost/casing should be found in the room or the immediate area the day after bag/block removal.

Final Aside: In closing I have found the bag/block method of mushroom growing fascinating. It is the lowest investment, lowest technology system there is, but with reasonable systems and good management it can be as productive and produce as good quality as any other system (Figure 8.18). In some areas, the labor issue can be exclusive of this cultural system, but in many other areas this can be an advantage. Additionally, if the operation in question makes its own phase I/II compost it becomes vertically integrated, which makes it very advantageous. I've worked with many bag or block operations over the years and find them challenging because each is unique. Whether it is layout, air movement, bag/block size, stacking, HVAC systems, labor, whatever, there is always a challenge. Equally if the farm is designed with reasonable systems there is always a solution. Having said that I've advised farms that have consistently produced 35–40 kg/m^2 on three flushes with great quality. As they say, there are "horses for courses" and there are many circumstances that are ideal for a bag/block *Agaricus* mushroom farm.

Figure 8.18 Harvested mushrooms.

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9

The Mushroom Industry in the Netherlands

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As far we know, in the Netherlands the first mushrooms (*Agaricus bisporus*) were being produced in 1828 not far from Amsterdam. In 1890, a book on mushroom cultivation was written. In 1889 mushrooms were being produced in caves in the southern Netherlands in the city of Valkenburg, in the cave named *Fluweelengrot*. Before the Second World War, in 1932, there were vegetable and fruit growers in glass houses in the western Netherlands with low rates of mushroom production.

These growers had many problems with disease, and before the war mushroom prices were very low, meaning these growers had limited results. However, there was a vegetable research station also researching mushroom cultivation from 1936 until 1951. That research station (Proefstation in the city of Naaldwijk) was testing the warming of compost in rooms for pasteurization purposes in order to remove diseases, larvae, eggs from flies and so on, in 1942. Production at that time was around 0–5 kg per m^2. They realized that some gypsum in the compost produced better results and that chicken manure would be a good raw material to mix in with horse manure.

In 1936 this research station made their own spawn with good results. After World War II, in 1945, there was a strong increase in mushroom production and prices in the market were very strong. Production at that time was very low per square meter, but mushroom prices were high at 10–12 guilders per kg (the guilder was the currency used in the Netherlands in that time), which equates to roughly €5 per kg. For that reason, in 1948 there were already around 50 mushroom farms in the caves of the southern Netherlands.

The reason that caves were interesting for growing mushrooms was down to their cheap location and the fact that mushrooms don't need sunlight to grow. After WWII, Dr. Bels and Dr. Bels-Koning were married and highly educated biologists, who started researching mushrooms in the southern Netherlands with financial support from the Dutch government (typical in Dutch culture was the financial support from the government, which was one of the pillars stimulating very strong development in the Dutch mushroom industry). Production at that time was low at 14–40 kg mushrooms per ton of fresh compost (phase I complete compost) over a long growing period. Currently (2015), production in the Netherlands is between 400–500 kg mushrooms per ton of fresh compost (phase I complete compost), which shows the very strong development over the last 70 years!

In 1950 came the first "professional" mushroom houses in the village of Mook and soon after in the villages of Horst and Grubbenvorst in the province of Limburg; the southernmost province of the Netherlands. At that time, the first growers started mushroom cultivation in rooms

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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Figure 9.1 Using compost blocks in shelves.

with concrete beds stacked 4–5 beds above each other. This is the typical Dutch one-zone farm design from that time, which means phase II, incubation, case run, and pinning plus harvest happened all in the same space (room).

Many years later, these concrete beds were changed for wooden beds and more recently to galvanized shelves. A *modern* Dutch mushroom farm has aluminum beds and this Dutch system is entering more and more professional farms worldwide (Fig. 9.1).

In 1953 the very important Mushroom Cooperation was set up with the name Coöperatieve Nederlandse Champignonkwekers (CNC) Vereeniging: in English, this means the Cooperative Dutch Mushroom Growers Association. With this cooperation, the Dutch mushroom industry developed very strongly making it the knowledge leader for mushroom cultivation in the world. In our lifetime, there has been strong development worldwide stemming from Dutch knowledge. The CNC was the organization that started with cooperative compost production; they stimulated strong united mushroom sales that is another pillar of strong industry development because the Dutch growers didn't see each other as competitors, which stimulated knowledge exchange in study clubs.

Each grower let other growers look at their farms, which stimulated very rapid progress. From 1950 to 1955 it was ITT (Instituut voor Tuinbouw Techniek) (Institute for Technology Development of Vegetable, Fruit and Flower Production) in Wageningen that developed optimal mushroom farms for growers. At that time, much research and intense discussions into the best mushroom cultivation systems took place, whether it was best to use a one-zone or multiple-zone system. Multi-zone systems involve transporting trays of compost from one room to others, depending on process stage.

Phase II happens in special pasteurization rooms. After pasteurization, the trays must be transported to the spawning line for spawning and afterward are transported to incubation rooms. After incubation, the trays are transported to the casing line (same line as during the spawning process) and after casing to the case run and pin-heading rooms. From there the trays are transported to harvest rooms.

The whole process finishes in heating chambers that cook out the batch to protect new batches against infections and disease. The consequence of this multi-zone system is the huge transport required and risk of easily damaging wooden trays. A negative factor is the small space in between trays that creates suboptimal cultivation control. The single-zone system is expensive for investment but overall more efficient and better for high production and good quality. The costs are lower overall in single-zone systems because of relatively fewer labor hours and more consistent production. Both systems have advantages and disadvantages, but gradually, these days the Dutch single-zone system seems to be the best for optimal mushroom production with the best quality in the world.

Two people started to help Dutch growers with initial cultivation advice and one of those was Mrs. Vaandrager who, some years later, helped Mrs. Bels-Koning in the new Dutch mushroom research station. In the period 1947–1952, the first short course on mushroom cultivation was held in the southern Netherlands.

In 1953 mushroom production in the Netherlands was more or less 300 tons per year. But sales of mushrooms were a problem leading to low prices. In that period, several growers realized that strong cooperation was the best solution to control sales better and to work together for compost and casing production.

November 20, 1953 started a new phase in the Dutch mushroom industry with the founding of the CNC. Mr. L Pijnenborg made this step and at that time was the director of the *Nijmeegse groenten veiling*, a cooperative local sales company for vegetables where mushroom growers also brought their produce. The main reason for creating the CNC was the poor position of mushrooms in the market, because growers at that time believed sales companies were unfair in the prices they were paying to producers.

The CNC started with eight members. Slowly, more growers became interested in this organization and they created a structure of several divisions depending on the location of their farm. Acceptable prices for mushrooms continued to be a problem and the CNC tried to introduce a price structure by creating an agreement that all members bring their mushrooms to one sales company, "the Veiling"; several members did not agree and left the cooperation.

In my opinion, the situation now in the Netherlands with low mushroom prices for the fresh market exists because many growers started too late in building up their own market, like the rest of the world.

Most of the Dutch production facilities don't have their own market. CNC growth in the 1950s was very rapid and in more Dutch areas new divisions were set up, and it became desirable to be a member of the CNC. Mr. L Pijnenborg, founder and member of the CNC, initiated the idea that growers need technical support. The Bels were invited by the Dutch government to start their mushroom research again, as they had done several years before. By the end of 1955 they came back from Canada to start again in Rhenen, the Netherlands, close to Wageningen, where some people were already active in development of mushrooms in the Netherlands with small research agencies and consultancies to support growers.

At that time the Netherlands had around 180 growers, and production was around 1100 tons per year. Dr. Bels and Dr. Bels-Koning started collecting all the knowledge gained so far in their own mushroom research book. In 1956 the Dutch government stimulated the mushroom industry to start their own *Mushroom Research Station*. There was one stipulation that the government would provide 50% financial support if the growers also paid 50% of all costs.

Growers found other financial support from the Veilingen (central sales companies, local provincial governments, and different local divisional governments). Dr. Bels was invited to be the first director of the Mushroom Research Station in Horst and government consultant for the mushroom industry in the southern Netherlands. His wife, Dr. Bels-Koning, worked for the government as a researcher with Mrs. Vaandrager as her assistant.

In 1957, Mr. van Soest, fruit and vegetable consultant in the central Netherlands, came to work for the Mushroom Research Station solely as a mushroom consultant. Both Bels and van Soest each took 50% from Dutch growers for their cultivation consultancy roles. At that time (1957), there were already 360 active mushroom farms. Mushroom production increased to 1500 tons per year and production reached 4 kg mushrooms per square meter. Both Bels and van Soest investigated in their own regions of the Netherlands into the best results from successful growers, and then informed other growers on what to do to get good results.

Total production in the Netherlands grew strongly and increasingly the CNC tried to make collective sales and the sales companies (De collective Veilingen) invest in more marketing. This was necessary because in just 1 year 100 new farms appeared and production increased at 1000 tons per year. The CNC organized a base price and all remaining mushrooms were stored.

A glut of these mushrooms seemed impossible to sell and there was no other option but to *destroy* them. The same happened with dried mushrooms because although the CNC tried different sales approaches to stimulate sales even that was not successful. The CNC had no other method to reduce the sales price and they advised growers to stop production in the summer months to reduce storage.

In the mushroom village of Kerkdriel (the American Kennett Square in the Netherlands), the first *mushroom school* was opened in 1958. The Mushroom Research Station supported these early mushroom schools in providing training. In the first few years after the research building was completed, there were many good results from testing and, together with strong team cooperation from the research station plus practical experience from mushroom growers, other growers gained excellent knowledge of mushroom cultivation and growth from the two mushroom consultants.

In that environment, Dutch growers felt a very strong connection with the research station. In 1957 the research station began the mushroom magazine *de Champignoncultuur*. In this, they published new research results plus a lot of practical tips to help mushroom growers improve quality from their cultivation. The magazine, *Mushroom Business* is, in principle, a later generation of that starting magazine. Back then the Horst local government along with ITT (another institute for Agaric technology also developed with financial support from the Dutch government) developed a new design for an optimal mushroom farm with single-zone culture beds.

With that action the multi-zone tray system lost favor in the Netherlands. After that all mushroom farmers followed this standard plan. The number of growers in that time (1959) increased to 500 with a total production for the year of 2500 tons.

The production per square meter increased to 5 kg per m^2 and prices increased as well to 2.70 guilders. In 1960 the development of steam casing the soil at a central location occurred; it was a challenge for growers to mix casing uniformly because it was an unpopular, heavy task.

Many growers were very positive and jointly invested in a central casing company by CNC. In 1961 casing production increased to 200 m^3 per week. A year later it became necessary to increase building and plant because the growth in casing production made it necessary to make two different casing qualities, because different growers had different uses for mushroom casing soil.

Many growers back then worked with blond peat. A logical choice, as growers knew that compost quality was good back then, unlike now. Then growers made phase II compost in rooms and now every grower in the Netherlands fills with phase III compost that is incubated in tunnels. In 1961 the research station got help from two new researchers; Mr. van Gils as mushroom consultant and a biology student, Mr. Gerrits, finishing his PhD studying the microbiological process during the fermentation of mushroom compost.

After Gerrits finished his studies successfully, he was the specialist in compost research for production up until his retirement. Following the first tunnel development in Italy, he developed more detail about the tunnel process for the Dutch mushroom industry. In 1962 Bels and Bels-Koning traveled to the USA to visit many farms with special compost facilities. They took many photos of their equipment. CNC consultants traveled to England to visit compost facilities there, because of the good results of a cooperative casing company there that interested the CNC, and because more growers were interested in centralized compost production.

Up till that time, the CNC only bought raw materials centrally so that growers could mix these into their straw. The plan was to start with production of 500 tons of fresh (finished phase I) per week. The first step of raising a 500,000 guilder investment was not easy for members of the cooperation, because many of them saw problems in large production and others were afraid that the CNC would not make the same quality of compost. Other members thought that 500 tons of phase I compost was too big a start.

At that stage, the central office of Tuinbouw Veilingen (central sale companies) created a better strategy for mushroom sales in Germany and the CNC invested in more marketing. In 1964 the official Dutch Mushroom School started under the guidance of P.J.C. Vedder. Initially, they gave the training in the research station. The hunger for mushroom knowledge was great because in one year, near the mushroom school, there were nine other training centers in the Netherlands with a total 250 people following the course.

For the research station, Mr. van Geyn entered as new mushroom consultant to support his colleagues during the strong growth phase. From Belgium they invited Mr. Oversteyns and the CNC asked him to help them to set up an optimal composting process. With research, education, consulting support by the Dutch government, and strong cooperation, the Dutch mushroom industry grew immensely.

The central compost production by CNC was successful and even with negative comments from several members, there was always the director, Mr. Ambrosius, who placated members by explaining the situation along with the changes that had to be introduced for improvement, plus he launched many proposals for increasing compost production. Ambrosius was like a Godfather at that time and without him the CNC would have been less successful than it was and is now. He was the oil in the CNC organization.

In the meantime, the number of farms grew in the Netherlands to 750 with a yearly production of 9 million kg per year. In 1965 Mr. L Pijnenborg retired. Without this man as well, the Dutch mushroom industry would never have been what it is now. The CNC produced compost in the traditional way in the open air on piles from 1963–1967. In 1967 the CNC decided to install a large roof over the compost yard to protect it, particularly against the Dutch rain (Figure 9.2). The CNC decided to buy a central spawn plant for all members. Together with that, many Dutch mushroom farmers increased their farm sizes and sales from phase I compost and casing increased.

In 1968, the CNC started to fill phase I with members' compost (Figure 9.3). Thilot in the village of Lottum produced this equipment following the example from the American filling systems still productive in the US mushroom industry today. CNC realized that cooperative working such as these filling systems is necessary for better competition with other European countries and creates an easier growing process.

Figure 9.2 The huge roof construction over phase I compost piles for many years. In the same place now are modern tunnels for phase II and III.

Figure 9.3 Many years before the development of tunnels, the CNC delivered phase I compost to all members in the Netherlands. The CNC filled these as well and many years on, they empty the rooms too.

By making it easier for growers, they motivated more growers to expand their farms or stay growing and not stop. In 1968–1969 the CNC bought the partnership from the mushroom sales company NV (Netherlands Champignons), in order to gain more influence in sales promotion. Also at this time, Bels retired and van den Pol became the new director of the Dutch Mushroom Research Station. The new Mushroom School Facility was built in Horst including an instructional mushroom farm. The four consultants, van Soest, van der Geyn, van Gils, and Klaver each got their own division of the Netherlands.

Importantly, the CNC bought the mushroom sales company Lutèce that at the time was suffering financial problems. Now, Lutèce is the biggest mushroom canning operation in the world. The main reason the CNC bought Lutèce was that it had a considerable influence in the Dutch mushroom market. The Dutch sales companies Centraal Bureau voor Tuinbouw Veilingen, Centraal Verkoopbureau, and the Veiling C.V.V in Grubbenvorst (now Veiling Zuid Oost Nederland) were the other members of Lutèce.

Twenty-five years after starting CNC in 1953 there were already 1000 mushroom farms in the Netherlands producing 25 million kg per year. After 9 years of CNC casing production, it decided to make a new casing facility because the old casing farm had reached maximum capacity and the market was still growing by 1500 m^3 per week. At the same time, the Dutch mushroom industry built a new research station in Horst-America (a village near Horst). In 1971 L. Pijnenborg's son, Jan, became adjunct director of the CNC. The production of phase I compost was already 4500 tons per week, all below a big roof.

In the same year, CNC started AMCO B.V. to buy and transport all raw materials such as horse manure, straw, chicken manure, and so on. The reason for starting AMCO B.V. was to enable one supplier to influence the greatest amount of raw materials delivered. In buying AMCO B.V., the CNC removed much of the influence of inflated raw material prices. The CNC was the biggest customer for Thilot, equipment builder for mushroom compost production in the Netherlands, and to benefit both, CNC and Thilot decided that CNC should buy part of Thilot. In 1971 BJP Ambrosius stepped down as chairman of the CNC due to taking on other work, so the CNC grew bigger and slowly became more complicated.

Ambrosius concentrated on developing the growing processes of the CNC. Veeken, a mushroom grower from the village of Standaardbuiten, took the chairmanship over. In 1972 Dutch mushroom production increased to 40 million kg per year and slowly growth from the industry reduced. In cooperation with IMAG in Wageningen, the CNC tested emptying beds with nylon nets to make the process much easier and faster. In the same period, the CNC bought another canning operation; Reulen canning in Herten, the Netherlands.

In 1973 the CNC started CAC as a new mushroom trade center to get a better open view of what was happening on the international mushroom market. Sales of mushrooms continued to be difficult for the Dutch industry. In this organization, the Reulen canning operation and Lutèce, the Netherlands, were a big part. In this period, the CNC started a compost emptying company emptying used compost from mushroom farms. At that point, mushroom prices were dropping slowly but continuously.

For that reason, more growers stopped producing mushrooms and so the square meters used in mushroom production dropped too. As the CNC was selling less compost and casing, times were tough financially. In 1975 there were big problems getting enough compost raw materials because of the very dry period Europe suffered then; this is a problem that recurs from time to time even now. Back then, this had the consequence of increased compost prices.

For the CNC, it was a hard time because together with all these problems, action groups for a better environment were creating pressure to do something about the unpleasant odors around compost facilities. Also, use of casing raw materials from the Dutch environment was no longer allowed because these groups also saw that as damage to the environment. From then on, the CNC started to buy raw materials from north Germany. The peat they found in Germany was even better than the raw materials from the Netherlands, which had to be mixed with sugar beet lime so the water absorption capacity good enough.

In 1976 there was a positive change in the Dutch mushroom industry: European political protection from mushroom imports from outside Europe helped to increase the price slowly. Spirit entered the industry by the development of tunnels and the production of phase III compost (Figure 9.4), plus the new harvest technology of machine-cutting mushrooms for the Dutch canning industry.

Ambrosius stepped down as chairman from the Dutch mushroom research station to concentrate on completing the new tunnel building(s) with the CNC. In 1977 the mushroom school (CCO), became a separate foundation and Veeken became the chairman of the CNC after election to chairman of the CCO. In that period, there were 900 mushroom farms in the Netherlands with production of more than 45 million kg per year. The CNC

Figure 9.4 Head filling machine for filling phase III compost and casing together in modern Dutch mushroom farms.

produced 5500 tons of fresh compost per week. Interest in the Dutch mushroom industry was great compared to the rest of the world and many international growers visited the CNC, mushroom farms, and more people from over the world followed training from the CCO in Horst.

For many years, the CCO was the world's mushroom school, with the possibility of showing theoretical and practical circumstances in instruction. All Dutch mushroom farmers followed the special "Dutch Champignon Vakschool" training meaning they worked on their own farm and over 2 years, visited the CCO 1 day per week to follow lessons (including myself). Mr. Vedder left in August 1983 after 20 years working as director at the CCO.

From 1978 the use of supplements in phase III compost started in the Netherlands. Growers using this supplement used it only after incubation of the compost. However, by the introduction in 1990 of phase III compost and incubated tunnels, the quality of the phase III compost was much more consistent and supplements more evenly distributed through the compost during filling to mix inside the head filling machine. From 1990 on, there was an increase in using supplements for phase III compost because of its growing availability.

Near CNC there are two other compost facilities that produce phase III compost for growers in Europe. Initially, the Theeuwen brothers in Blitterswijck (also in the southernmost province of the Netherlands) started a phase I compost facility. Many years later, van Walraven and Kroon bought the Theeuwens' compost facility. They changed the name to Walkro. This company has one location in Belgium as well as in the Netherlands; even bigger than the CNC.

Another compost facility is Hooymans in the southern province of Brabant and in the other mushroom growing area, Kerkdriel, in the Netherlands. Near these three compost facilities, all having an indoor phase I facility that does not produce odor and is controlled by the Dutch government, there is another big mushroom company owned by the Peffer family, that has its own indoor phase I facility and phase II and III tunnels for the production of phase III compost

(Buth, 2004). Near these complete Peffer production facilities they have their own canning factory named Prochamp. These facilities are all located in the same village of Kerkdriel.

Once again, the mushroom industry in the Netherlands declined because of poor mushroom prices. There was too much competition from production in Poland and China. In 2004 there were only 350 mushroom farms left in the Netherlands. The production in 2004 declined further again and is now at 6%. However, production in the Netherlands has increased by 3% to slightly reduce this bad situation. By using shorter growing cycles 3% more production is created as this creates only two breaks in phase III compost, meaning a 4–5-week schedule for growing and harvesting. Continual increases in compost, energy, and labor costs were negative influences. Looking back from 1990 to 2004, it was 2004 that produced the worst financial results.

Now, in 2016, there are only around 100 mushroom farms left in the Netherlands. Square meters per company grew rapidly because mechanical harvesting farms increase production greatly due to a better mushroom price/cost ratio and the industry produces a lot of mushrooms for the frozen industry. A lot of that product is exported to the USA because the Dutch, with their very efficient mechanical harvesting systems, produce cheaper mushrooms than the Chinese.

Many companies that produce mushrooms for the fresh market buy or rent other production facilities from growers who have stopped production. With very modern Dutch mushroom farms, the whole process is well under control. Computers control the process in detail. An example is the deficit control developed by Jan Gielen from Cpoint that controls continual evaporation, even when the outside air humidity is very low or high, via a specially developed chip. Modern head filling machines measure the weight of the compost constantly ensuring that all areas in a room have the same amount of compost. Automatical watering trees using computer control can give water 24 hours per day; just what the grower wants and what the process needs. Automatic picking lorries move slowly but continuously beside the shelves so that laborers move constantly to pick only the biggest mushrooms.

Several times a day the lorry passes with the picker at the same places, meaning other mushrooms increase their weight by growing and at the moment they need to be picked, the lorry with the laborer is back again to pick the bigger mushrooms (Figure 9.5).

These details increase production with an intelligent way of picking. Development of mushroom production and harvesting does not stand still. In the Netherlands, several companies are working to develop better harvesting systems. Scanning the mushrooms on the beds and calculations bring laser light on the mushrooms that need to be harvested. The picker no longer needs to think about which mushrooms need picking (for more information about harvesting, see Chapter 13).

With that, production increases again. Other companies are working hard to develop robots that can pick mushrooms 24 hours per day. The time is coming closer that this dream will be a possibility. There are some one-bed systems with high cutting technology that increase picking greatly to more than 100 kg mushrooms per hour, per person. A strong development is use of modern technologies that reduce energy costs by more efficient use.

New equipment is available that splits casing and compost when emptying the beds. Dutch mushroom growers have to pay high prices for dumping their spent compost. To take out the spent casing reduces the volume and weight of the spent compost, which reduces the final cost of spent compost removal. Also, research has developed bed cooling, meaning that they can cool the compost directly in each bed. This means that higher filling weights and/or more supplements are allowed because there is more direct compost temperature control; this can increase production greatly by using more compost. With solar panels on roofs of mushroom farms, there are farms that produce their own energy. Burning of a farm's spent compost to

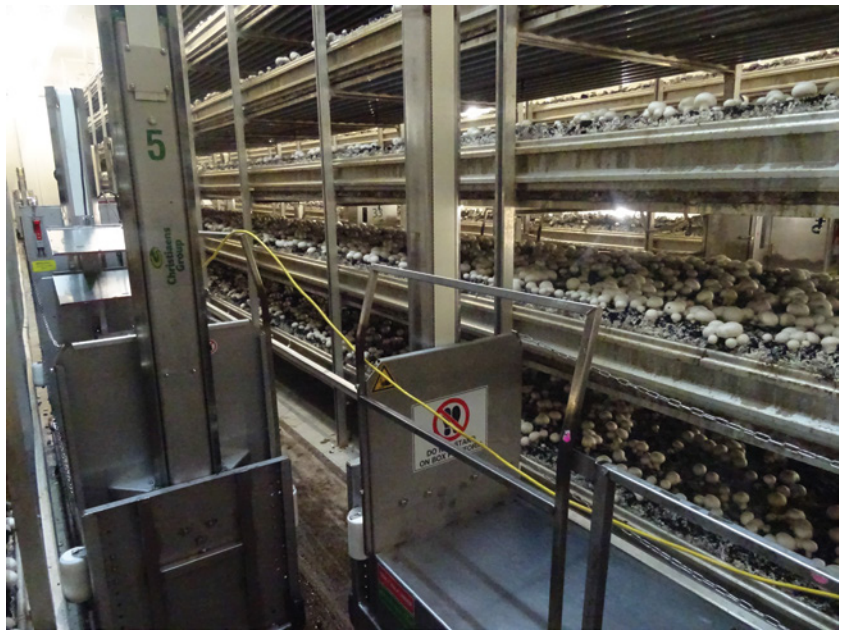

Figure 9.5 Automatic lorry: the yellow cable follows after picking to connect the batteries.

produce its own energy is another development. In the Netherlands, we call it the mushroom farm of the future.

The CNC is still active today with huge compost production from more than 9000 tons of phase III compost per week for Dutch and international customers. The CNC produces their own phase I compost in an indoor facility in Moerdijk (an industrial area close to Rotterdam) for making this large amount of phase III compost. The CNC has two different phase II and III locations, Moerdijk near Rotterdam and Milsbeek in Limburg – the southernmost province in the Netherlands near the German border (Figure 9.6).

At the start, the CNC had only eight members and the first chairman, B.P.J. Ambrosius (who did a lot of good work during his career for the Dutch mushroom industry as director and chairman of the CNC), never had any idea how important their work was for the whole Dutch mushroom industry, or for the whole international mushroom world. Sales were and still are a big point of attention for acceptable prices. By looking to other countries just in terms of sales, the Dutch mushroom industry makes, in my opinion here, an historical mistake. The Dutch mushroom industry has always concentrated strongly on production efficiency and so many growers didn't build up enough of their own sales, unlike in other countries. In many countries, each mushroom farm has their own customers that makes the price better for growers. Now, we see in the Netherlands the consequence of a suboptimal sales strategy for mushrooms.

Some big sales centers for fresh mushrooms in the Netherlands make or break the prices now. They make also the big money with sales and not the mushroom growers. This is a strong contrast with mushroom farms in the rest of the world. But the cooperative working of the Dutch mushroom industry and the role of sponsorship from the Dutch government in history, and still now for education and research, makes mushroom production in the Netherlands so strong!

The history of the Dutch mushroom industry was created in the southern Netherlands (Buth, 2011). This history still influences results in modern mushroom farms (Figure 9.7) with

Figure 9.6 Indoor compost facility of the CNC. The CNC also has another location containing many tunnels. The plan is to develop an indoor facility there as well.

Figure 9.7 Typical Dutch mushroom farm in 2016.

Figure 9.8 In the filling hall you cannot see compost conveyer belts because the input belts are above the ceiling and the output belts below the floor. The tunnels are filled completely automatically, with no person in the tunnel. There is only one laborer watching a monitor to control the filling process.

metal shelves, mechanical harvesting, and the possibility of developing robots for fresh mushroom harvesting.

Modern tunnel facilities and (indoor) modern phase I facilities, including computer controlled systems and complete clean controlled biofilter air cleaning systems, have been developed (Figure 9.8).

Rules and laws, plus low mushroom prices, make it so difficult for the Dutch mushroom industry but with power and creativity plus innovation, people in this industry are the winners despite negative influences over many years on the whole. Use of nylon nets to move the compost with casing mechanically into the beds is done by Dutch equipment (Figure 9.9).

Modern tunnels were invented in Italy and further improved in the Dutch Mushroom Research Station in the Netherlands. Indoor composting, developed by the CNC and the Dutch Mushroom Research Station, plus optimal climatization with help from Jan Gielen from Cpoint, are the reasons why the influence of the Dutch is so powerful in the world today. Another indoor phase I facility is planned for the village of Milsbeek.

Mushroom production in 2015 in the Netherlands was 260 million kg produced by 120 mushroom farms. Near the CNC, there are other compost facilities. In the following overview you can see the current weekly phase I consumption for tunnel facilities in the Netherlands and Belgium.

1) CNC in Moerdijk produces 16,000 tons phase I compost per week.
2) Monaghan, with three facilities in Blitterswijck, the Netherlands, Maasmechelen (Belgium), and Maaseik (Belgium), has a total production of 18,500 tons phase I compost per week.

Figure 9.9 An actual situation in the Netherlands where they fill phase III compost plus supplement and casing using modern equipment all together on a typical modern Dutch mushroom farm. In roughly 1 hour they fill about 200–300 m^2 with these materials. The organization is so good that the head filling machine (from a special filling company) plus the truck with compost and the truck with casing soil, from different companies, arrive at the same time. Just 90 minutes later, everything is gone and the room is filled perfectly.

3) Hooymans in Velddriel (the Netherlands) produces 4500 tons phase I compost per week.
4) Veco in Riemst (Belgium) produces 2500 tons phase I compost per week.

The next mushroom farms that have tunnels near production and that buy weekly phase I compost are:

1) Richamp in Velddriel using weekly 1050 tons phase I compost from Hooymans.
2) Agarica in Hoogeveen using weekly 400 tons phase I compost from CNC.
3) Bercvenne in Lierop using weekly 320 tons phase I compost from Veco.

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10

New Technology in *Agaricus bisporus* Cultivation

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10.1 Introduction

Generally, the mushroom production industry of *Agaricus bisporus* has become an increasingly competitive environment and there have been huge changes in how to produce more mushrooms efficiently and more economically. In recent years, new technology has developed creating more productive, commercial, and economic potential; as modern as it is effective.

This new technology, known as the "French system," is arguably an adaptation of Dutch culture, and was created as a combination of what at that time was taking place in France and Italy to increase and improve the use of space inside crop halls; the Dutch system has 4–6 levels of shelves (Klaver and van Gils, 1988), but this system has compost placed 10–12 shelves high. This takes more advantage of conditioned space and has a great impact on profitability given the high-energy cost of a controlled environment with little bed surface per square meter of floor area.

While more capacity is available for compost in the crop rooms, this new technology means that the substrate arrives at the facilities in Phase I, so that Phase II, and Phase III shelf filling, casing, and harvesting are all done under the same roof, in the same facilities, and using highly qualified personnel. This is critical to saving space while avoiding contamination risks during compost transportation. Figure 10.1 shows a plan of a mushroom farm.

10.2 Stages and Operations of the Production System

10.2.1 Compost Phase I, II, and III

The first step after the preparation of Phase I compost (see Chapter 6 of this book), is the arrival of this compost at the facility to continue with Phases II and III. Phase I is the first stage and the only one not developed within these facilities, since normally we get Phase I compost from modern composting plants nearby, which are responsible for the manufacture (Figure 10.2).

These compost manufacturing plants are equipped with the latest technology in construction, control equipment, and process development machines.

The process begins with the mixing and hydration of raw materials, mainly cereal straw, chicken manure, horse manure, urea, ammonium sulfate, and gypsum, which once mixed so as

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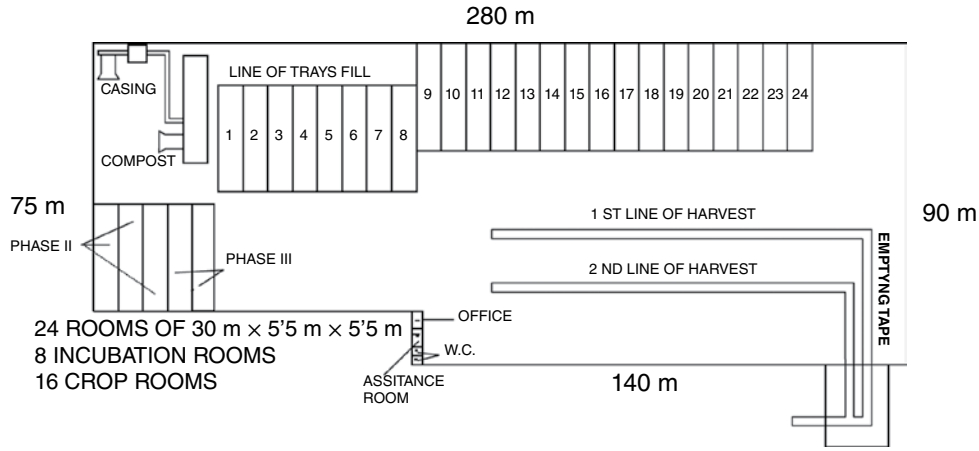

Figure 10.1 Plan view of a typical mushroom farm.

Figure 10.2 Modern composting plant in La Rioja (Spain).

to be as homogeneous as possible, are subjected to a fermentation process within so-called "bunkers." Due to increased microbial activity, the temperature of the compost can reach approximately between 70 and 80°C, trying not to exceed 80°C at any time. The process is controlled with auxiliary ventilation and oxygenation.

During the process, it is necessary to make at least two changes of material from one bunker to another, since the temperature of the compost begins to drop due to decreased activity by depletion of the microbial energy source, and in order to achieve greater uniformity of the whole mass.

Once fermentation or Phase I is complete, the compost is transported in trucks completely enclosed directly in the facility where pasteurization or Phase II will take place. Phase II composting is discussed in detail in Chapter 6 of this book. Basically, it consists of two stages, pasteurization for at least 6 hours at 59–60°C and subsequent conditioning between 48–46°C, and with a final compost temperature drop to 25°C, prior to inoculation with the mycelium. Both Phase I and Phase II follow the general criteria described by Fermor et al. (1985).

The duration of pasteurization and conditioning is around 6 days, after the tunnels are emptied (Phase II), inoculated with mycelium, and the tunnel filled again to proceed with colonization of compost in mass, known as Phase III. Usually, three Phase II tunnels are used to fill two Phase III tunnels. The ideal temperature for mycelia growth is 25°C and this temperature must be maintained for 14–16 days for the incubation process (mycelium run), while maintaining adequate ventilation at very low pressure, using air treatment equipment to regulate the speed and engine power for the fan as needed.

Once the mycelium run is completed through the compost, the filling phase of the mechanical tray line starts, to which is also added the casing layer and the first irrigation is applied. The trays are moved to the incubation room where colonization of the casing layer and subsequent fruiting occurs.

A second option after pasteurization is compost inoculation mycelium and filling of the cultivation trays with the same mechanical filling line, as opposed to phase III mass. Then the trays are transferred to the incubation room for at least 14 days with a controlled temperature of between 25 and 27°C and relative humidity of 95%, so that both the incubation phase and fruiting are held in strict climate-control special rooms (similar to the classic Dutch system).

10.2.2 Supplementation

Nutritional compost supplementation can be provided simultaneously with inoculation or after the compost been colonized and before applying the casing layer, or even in its own casing layer using a specific casing supplement in the same filling line trays.

To achieve the desired effect, supplementation should be used only when we have a good quality compost and it is well colonized (in the case of its application "at casing,") and is also essential to make a correct dose adjustment and homogeneous distribution of the supplement, to prevent overheating of the compost by overactivity, ensuring that at all times the temperature of compost does not exceed 30°C, a situation that could lead to the disappearance of mycelium both inside the compost and at the surface of the casing layer, facilitating the emergence of possible competitors.

It is also necessary to have a good independent spawn dispenser to avoid contamination and, of course, maintain strict hygiene, cleaning, and disinfection throughout the process.

Most supplements are based mainly on denatured soybean meal (Zied et al., 2011), although other sources of proteins, lipids, and minerals are possible; these proteins are often subjected to treatment with formaldehyde to provide sustained release of nutrients.

The amounts provided generally depend on the time of application and the analytical characteristics of compost (mainly its N content), the type of supplementation used, and the number of flushes that it is planned to collect (two or three flushes), since there are different types of supplements on the market with different amounts of protein for each need. As a guidance, the amounts normally used are:

To Phase II compost: between 0.80 and 1 kg per square meter of growing area.
To Phase III compost: between 1.5 and 2 kg per square meter of growing area.
The most widely used supplements in the European market are Promycel®, Champfood®, Natural Gold®, MC Substradd®, and SuperChamp®.

10.2.3 Trays

After supplementation with well-developed mycelia and fully colonized compost, it is introduced into trays through the mechanical filling line; the trays are made of galvanized iron to prevent corrosion, extend life, and avoid possible contamination that could remain in the holes made by the oxide.

The trays are 3.5 m long and 1.6 m wide, with a total height of 41 cm and a useful surface 5.6 m^2 culture. The trays are stacked 12 high above each other, leaving a 20 cm space between them.

The capacity of each tray is 500 kg of compost and each room has capacity for 200 trays, which gives us a capacity of 100 tons of compost per room. Before inserting the compost, each tray must have clear, perforated plastic placed at the bottom to protect and prevent moisture loss, but allow some gas exchange (Figure 10.3).

Figure 10.3 Filling tray with compost and casing on plastic film.

One of the great advantages of this new technology is that the filling of the trays with compost and the immediate application of the casing layer are carried out successively in the same process, which is performed in a completely clean facility, disinfected, and equipped with air filtration equipment to avoid any contamination, using a single line to put the compost and casing where ruffling is carried out and irrigation water is then applied (Figures 10.4 and 10.5).

At the end of this line, the trays are automatically stacked until they are moved with forklifts to one of the rooms for the development of mycelium in the casing layer. The trays can stay in the same rooms for cultivation, but can also go directly to external growers in other locations without waiting for development of mycelium before transportation.

10.2.4 Casing

The casing layer is of crucial importance in the cultivation of mushrooms, since it has a decisive influence on the type of mushroom fruiting and quality, determined by the management of environmental conditions, and also has a major impact on the yield of the crop.

The correct selection and management of the casing can make the success or failure of the crop (Chapter 7 discusses the casing layer in detail). Mainly, it is important that the casing meets the following requirements:

- Have a good structure: the more compact the structure of the casing, the fewer mushrooms develop, but are much heavier. The structure also affects gas exchange capacity and ease of handling.
- Be free from pests and diseases.
- Have a high water holding capacity.

For this new technology of *A. bisporus* cultivation, different types of casing, depending mainly on location and availability, are used. Some of the most commonly used in Europe,

Figure 10.4 Line filling the trays.

Figure 10.5 Detail of irrigation (indicated by the pale arrow) and ruffling (indicated by the dark arrow). (*See color plate section for the color representation of this figure.)*

Figure 10.6 Line of casing, conveyor belt, and mixing drum.

based on peats, are BVB, Euroveen®, Top-terra®, CNC® (Netherlands), and Harte Peat® (Ireland). Spain also uses another local casing based on peat (Infertosa®), while moving away from the mixtures of mineral soil and peat that had been traditionally used.

Although peat is the material most widely used as casing for mushroom cultivation throughout the world (Pardo-Giménez et al., 2012), the use of spent mushroom substrate as the main casing material may be possible for reducing production costs (Pardo-Giménez et al., 2011). In Chapter 7, a list of materials that have been used or evaluated as ingredients of casing layers in mushroom growing, either commercially or experimentally, is presented.

Another possibility to consider in this kind of cultivation is the "compost added to casing (CACing) technique," where compost Phase III is applied to reduce the duration of crop cycles and achieve a more uniform product (Zied et al., 2010).

The casing layer is applied over the compost to a height of 4 cm and constitutes a reserve of water throughout the crop, helping to keep moisture where fruiting occurs and provide water for mushroom development, serving as water supplement provided by the compost. The moisture content in the casing layer based on peat used is usually above 70% (see Figure 10.6).

10.2.5 Growing Rooms and Cultivation Cycle Management

The crop rooms of this system are built using sandwich panels with high density insulation and equipped with precise HVAC equipment to maintain a controlled atmosphere in terms of temperature, oxygen, carbon dioxide, and relative humidity, as well as air filtration. The whole air conditioning system is located under the roof on top of the camera (Figure 10.7); this space includes the environmental control equipment, which is also conditioned to the right temperature to avoid any variation inside the crop rooms.

The dimensions of the rooms are 30 m long, 5.5 m wide, and 5.5 m high, with the trays arranged according to Figure 10.1 and 10.7; they are 12 trays high and 16 rows deep. The rooms are attached next to one another with a sequence of 24 chambers of which 8 rooms are used for incubation and induction of fruiting, being separated and isolated from the remaining 16 rooms that are used for the crop (cultivation of mushrooms), to effectively avoid contamination during incubation and fruiting.

The overall design of the crop cycle is as follows: (1) During the first 6 days after application of the casing layer, the temperature of the compost is kept between 26 and 28°C, after which it proceeds to the induction of fruiting. (2) Induction of mushrooms is performed under the same incubation chambers once the mycelium has completely invaded the casing layer and having

Figure 10.7 Facility used for the new technology of *A. bisporus* cultivation. Incubation and crop rooms with the line of harvesting.

provided about 26 liters of water per m^2, while maintaining high relative humidity within the chamber, close to 100%.

Importantly, during this phase of mycelia growth in the casing layer the interchange of trays in the same incubation chamber occurs, since the mycelium does not invade all heights due to the effect of pressure and air velocity. Thus, the four top trays are changed for the four intermediate trays and four intermediate trays for the four top trays, to get mycelia growth uniform in all trays and thus achieve as uniform a mushroom harvest as possible. Once the mycelium has spread across the surfaces of all trays in the same stage of development, the induction of mushrooms (pinning) is done and after another 6 days, mushroom formation begins.

At this pinning time stage, the trays are transferred to the crop rooms where in the progressive lowering of climatic parameters for mushrooms formation happens. An example of this and the variables used is presented in Table 10.1.

After the first flush, irrigation of the trays is provided at the end of the line. This irrigation is about 6 liters per m^2, then the trays are introduced to another crop room until the second flush. Once the second flush ends a small watering is added, about 3 liters per square meter and trays are reinserted in another crop room for the third flush.

10.2.6 Production and Harvest

The harvest of mushrooms starts by taking trays from the crop room and moving them with forklifts to the start of each line, since it is normal to have two harvest lines where trays are

Table 10.1 Sample of managing environmental conditions for fruiting of *A. bisporus* for the fresh market.

Induction of Mushroom (Pinning)				
Day	**Air Temperature (°C)**	**Compost Temperature (°C)**	**Relative Humidity (%)**	**CO_2 (ppm)**
0	22	27	98	5000
1	21	26	95	3500
2	20.5	25	95	2800
3	19.5	24	95	2800
4	18.5	23	94	2400
5	18	22	93	2000
6	17.5	21	92	1800
7	17.5	20.5	92	1600
8	17	19.5	91	1600
9	17	19	90	1500

stacked in sixes. Continuously, the controller of the line begins to move the trays one by one and in line with a proper rate of feed depending on different factors such as the number of mushrooms of the quality desired and the skill of personnel involved in the collection (Figure 10.8).

Usually collection is done for 4 days for the first flush, 3 days for the second flush, and 3 days for the third flush.

It is important to note that this system is more complicated for performing various harvests to date on the same tray as in the Dutch system, since removing trays is a technically laborious operation that must be performed several times a day in each crop room. It is thus crucial that all trays present the mushrooms in the same state of development to avoid having to remove them to harvest only mushrooms where the cap is opening.

The temperature inside the crop rooms is around 18°C and is kept at 18°C for the harvest in the facility, therefore the mushrooms do not suffer changes and modify growth during the harvest.

After that the daily harvest trays are returned to the crop room. When the trays reach the end of cultivation through the line and their mushrooms have been picked, they are automatically stacked waiting for the "bobcat machine" to pick them up and deposit them into another crop room, made clean for cultivation, and disinfected for the next day to continue the harvest and finish the flush completely. The necessary irrigation is automatically provided at the end of the line through a shower system.

Another advantage of this collection system compared to the Dutch system is that harvesters do not have to walk up and down the stairs from the shelves, since they are at the bottom of the line-up and the trays displace through the line itself. It isn't necessary either to constantly manipulate the boxes with the mushroom harvest, since they are limited to placing them on a conveyor belt in front of them once they are complete (Figure 10.7).

The duration of the normal cycle is about 40 days. Another advantage of this production system is that the total time of production, including Phase II, Phase III, casing, pinning, and harvesting of flushes is 43 days, compared to 45 days in a traditional crop.

Figure 10.8 Different views of the harvest line.

10.2.7 Compost Discard

The ease of removing and unloading trays with spent compost is another feature of this system, since once the third flush of mushrooms is harvested, the tray travels across the line to the end, where it is mechanically emptied of all the compost. At the same moment, the empty trays are cleaned with pressurized hot water and transferred to a tank with disinfectant and immersed for disinfection before use again. The spent compost, once unloaded from the tray, is transported on a conveyor belt that leads to the outside of the facilities where the truck is waiting to remove the compost.

10.3 Conclusion

The system described in this chapter is a new development in the mushroom industry for efficiency and energy savings. The main difference that this new technology brings is that once the compost is received within the facility, material only leaves when absolutely all mushrooms produced are harvested.

The advantages of this system are:

- Easier to harvest.
- Greater ease of temperature control.
- Easier use of supplements.
- Less risk of contamination by flies.
- Increased capacity filling trays (kg/m^2).

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11

Insect, Mite, and Nematode Pests of Commercial Mushroom Production

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Mushroom production represents one of the best examples in agriculture where pests can be controlled without the use of chemicals. Because mushrooms are typically cultivated in a protected environment, direct control over its pests is quite achievable. The fundamental principles necessary to manage pests, diseases, or other molds are the recognition of the problem through its symptoms and signs and an understanding of the actual organism responsible, its life cycle, and spread.

In mushroom production, the principal pests are flies, mites, and nematodes. Other pests such as mice, spring tails, thrips, beetles, and sowbugs have been reported (Snetsinger, 1972).

This chapter will focus on pests associated with commercial production of *Agaricus bisporus* and their management.

11.1 Fly Pests

Commercial mushroom fly pests include three dipteran families: Sciaridae, Phoridae, and Cecidomyiidae (see Chapter 16, Figure 16.11). Each family can cause significant yield or quality loss and vector mites, nematodes, and diseases on commercial farms. The dominant problematic species display regional, annual, and seasonal variations.

11.1.1 Dark-Winged Fungus Gnat

Sciarids are commonly known as the dark-winged fungus gnats, sciarid flies, big flies, or mushroom flies. The predominate fly species in North America is *Lycoriella ingenua* (Dufour) (syn. *L. mali*, *L. solani*) while in the UK both *L. castanescens* (Lengersdorf) (syn. *L. auripila*) and *L. ingenua* are mushroom pests with *L. castanescens* being the more serious pest there (Menzel and Mohrig, 2000; Fletcher and Gaze, 2008). *Bradysia* spp. are also pests of mushrooms in other parts of the world (Fletcher and Gaze, 2008). Only *Agaricus bisporus* is subject to severe attack by this gnat but it also will breed on oyster and shiitake mushrooms. In general, the dark-winged fungus gnat can be found in greenhouses in both soilless and soil mixtures, in composting debris such as leaves, and outdoors in wild mushrooms.

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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11.1.1.1 Damage

Dark-winged fungus gnats can be found on any mushroom farm, but direct yield losses occur only when the gnats go unchecked. Larvae of this fly are general feeders, consuming mushroom compost, mycelia, spawn grains, mushroom primordia (pins), and carpophores. When mushroom primordia are small, up to about 1.5 cm diameter, the larvae can consume the entire internal contents. The mushrooms will appear glossy and light brown, and the small carpophore may be completely perforated and, when picked, the tissues crumble. Carpophores that are larger when attacked show black necrotic areas in the stipe where the larvae made feeding galleries. Some larvae do not tunnel into the stipe but consume the mycelia at the base of the stipe, in which case the mushroom does not develop normally. Generally, little direct damage from this gnat will be evident on first-flush mushrooms because the fly population has not developed sufficiently. However, second- and subsequent-flushes may show some damage from larval feeding. Kielbasa and Snetsinger (1980) established that 108 females per square meter were required at spawning to set up conditions for economic losses.

Perhaps its greatest impact is as a vector of mushroom pathogens, such as dry bubble disease (*Lecanicillium fungicola* (Preuss) Zare) and Gams [syn. *Verticillium fungicola* (Preuss) Hassebrauk, *Verticillium malthousei* (Preuss) Ware]) or green mold disease (*Trichoderma aggressivum* Samuel and Gams f. *aggressivum* and *T.a.* f. *europaeum*) (syn. *Trichoderma harzianum* biotype Th4 or Th2, respectively), from diseased to clean areas in the same production room or to clean crops on the farm.

11.1.1.2 Identification

Eggs of this fly (family Sciaridae) measure 0.25 by 0.15 mm and are smooth, oval, white, and translucent. Mature larvae are about 7 mm in length, have a white, translucent body and a black head capsule (Figure 11.1). Pupae are about 2.0–2.5 mm in length, are white at first but turn black prior to eclosion. Adult males and females measure between 2 and 3 mm in length; most often they can be found near a light source. The adult wing has a distinctive forked and cross vein.

Figure 11.1 Sciarid flies, adult. Credit: Photo graciously provided by F.J. Gea and M.J. Navarro (CIES, Quintanar del Rey, Cuenca, Spain). (*See color plate section for the color representation of this figure.)*

11.1.1.3 Life History

This fly usually invades the mushroom crop at or near the time of spawning. This can occur in single or multi-zone systems. After invasion, adults may oviposit on mushrooms, on compost, or in the casing soil, laying eggs singly or in small groups. The larvae have four instars and may feed on the spawn grains, mushroom compost, mycelia, and mushrooms.

Within four hours of emergence, female gnats are sexually receptive and usually will have mated. Egg-laying begins soon afterward. Female flies are strongly attracted to odors emanating from the mushroom facility, particularly as the compost cools after the pasteurization and conditioning phases. Peak fly-invasion generally occurs within four days of spawning. Flies always seek the nearest site for oviposition; in commercial mushroom operations, this is usually nearest to doors of the production facility. Larvae developing in the surface layer of compost, which is later to be cased, will move through and pupate at or near the surface of the casing layer.

The development times from egg to adult, survival, and fecundity are temperature dependent. The optimum temperature for development and maximum survival is 18.3°C. If flies enter at spawning, then the first-generation adults will emerge about three weeks later. At higher or lower temperatures, mortality increases; it may be as much as 33% at 27°C. Female fecundity is inversely affected by temperature, such that an average female will lay 160 eggs at 12.8°C, but only 63 eggs at 26.7°C. (MacDonald and Kielbasa, 1977; Kielbasa and Snetsinger, 1978). The biology is not only dependent on temperature but also on the species on which *L. ingenua* is feeding (O'Connor and Keil, 2005).

The number of generations on a mushroom farm depends on the mushroom crop length. Multi-zone systems harvesting only two flushes may only have one complete generation during each cropping cycle. However, if the crop is extended beyond two flushes then two or more complete generations are possible.

11.1.1.4 Management Strategies

The movement of sciarid flies from one production room to another or from one mushroom farm to another is accomplished mainly by unassisted adult dispersal and the persistence of the insects, which will crawl through any crack or crevice into the mushroom production facility. In temperate climates in the winter with outside temperatures below 7°C, sciarid populations are lowest since migration and outside reproduction is limited due to temperature. Although the threat of fly problems is minimal at this temperature, sciarids can move from one production room to another through corridors, lofts, mezzanines, or outside the building; and they have been seen on the surface of snow 7–10 m away from production rooms where they have emerged. During warmer months, they will move from building to building, and to farms several kilometers distant.

Under conditions of poor sanitation and hygiene, eggs, larvae or flies can enter the room on equipment that was not cleaned from previous spawning or casing procedures. In addition, by leaving spawn overnight in a fly-infested corridor, an infestation of flies can be introduced into the production room through the spawn or boxes. Growers who obtain mushrooms from other growers run the risk of introducing fungus gnats along with diseases, if other farms have a fly problem.

1.1.1.4.1 Monitoring

When an invasion occurs, the size of the initial population and predicting its future size are important considerations. Monitoring gnat populations provides this information, but such factors as climate, disease, immature and adult insect populations, and growing practices are also important (Wuest and Bengston, 1982).

The adult stage of the dark-winged fungus gnat is the main concern when monitoring on a commercial mushroom farm. Monitoring for adults is accomplished by means of a fluorescent or black light as a fly attractant and a sticky surface to trap the flies (Wuest and Bengston, 1982). Yellow sticky cards are less attractive than a light source.

All production rooms should have a monitor regardless of farm system. Some monitor locations within the room may capture more flies than another. In a single-zone shelf farm or in phase II tray system, the monitor should be placed in the room prior to the compost cool-down period when air temperature reaches 43°C. Monitoring is more challenging for multi-zone farms with phase II and spawn-run tunnels. If the tunnel is totally enclosed in a building, which is the most desirable, a monitor should be placed in the work area. Some farms have placed monitors inside spawn-run tunnels at filling for monitoring during spawn-run. Monitors positioned outside, where flies normally roost, and in the picking corridor, provide an indication of the background or endemic population level and measure the effectiveness of an outside or corridor control program. In cool weather, the picking corridor may serve as a significant bridge for fly movement between rooms.

Accurate fly catches should be recorded daily to evaluate control programs and to design future strategies. Threshold levels at different stages of a crop vary. For this reason, growers are encouraged to determine their own economic threshold levels. On single-zone farms, one fly on a monitor has been considered a sufficient threshold at spawning for action.

11.1.1.4.2 Cultural Practices

Multi-zone systems in North America are harvesting for only two flushes. Shortening cropping cycles have been demonstrated as a viable economic management practice and ancillary to this fly and disease problems are reduced. Rapid cool-down at the end of phase II reduces the time available for fly invasion. Higher temperatures occur in the compost during spawn-run and after casing, compared to the harvest period. Shortening the spawn- and case-run prolongs fly emergence in the cycle of the crop's phenology. In general, less fly problems will occur if the spawn-run is short.

Fly control at the end of a crop is just as important as control during spawn-run. Growers should heat-treat the old compost at 60 to 65°C for 8–12 hours to kill flies at all stages of development; these conditions will also kill most disease-causing fungi and bacteria. (Note that this temperature and duration is not sufficient to eradicate virus disease and especially green mold disease (*Trichoderma aggressivum f. aggresivum*).) A crop may have to be terminated earlier than the schedule dictates to ensure that the population of emerging flies can be controlled prior to its spread to other locations. Prior to adding heat, an adulticide should be applied to eliminate adult fly escapees.

Prevention is the most effective way to control fungus gnats. If adults can be prevented from entering the growing rooms, then the problem is solved before it begins. Cracks in walls, and around air conditioners, pipes and doors, are the usual routes of initial fly invasion. In one-zone systems, installation of fly netting over doors during spawn-run has been helpful. In all systems, limiting the amount of traffic into the room at anytime, can help reduce the likelihood of infestation. Monitors can be used to determine the tightness of a room and the need for doorway management. In general, if flies can be excluded through the time of casing, they will have little or no direct impact. However, if there is disease on the farm, then the flies will vector this into rooms at whatever time they enter.

Good sanitation is also important for fly control. Flies can breed in the stumpage and fragments of discarded mushrooms, and spent compost may serve as breeding material. Spent compost and mushroom stumpage should be removed from the premises. Growers should also remove and dispose of trash promptly.

The mushroom farm community must also be considered. Each room, block, and farm has an endemic or background population of fungus gnats. These populations are specific for each farm and will vary from crop to crop and season to season. Pest populations throughout the farm community can only be brought under control if each grower understands the benefits of a consistent and total fly control program. Cooperation among growers also promotes better fly management, based on sharing of knowledge about fly biology and behavior, and the essential conditions that favor colonization and spread of disease pathogens.

11.1.1.4.3 Biological/biorational Control

Biological and biorational products are used to control sciarid flies. These manage specifically the intended target, having little effect on humans, non-target organisms, or the environment. There are several larvicides available. Larvicides are most efficacious and efficacy is affected by the life stage of the larva at application (Rinker, 2002). *Bacillus thuringiensis* var. *israelensis* and diflubenzuron tend to be more effective against younger larvae, while entomopathogenic nematodes, especially *Steinernema feltiae*, and methoprene are more effective against the older larvae. Timing of application should rely on monitor counts. Application of benomyl may reduce the effectiveness of the *Steinernema feltiae* (Atawa et al., 2013). Cyromazine and diflubenzuron modify the development of the exoskeleton of the larva while azadirachtin and methoprene, as insect growth regulators, mimic the naturally occurring hormones in the larva's body, interfering with the natural growth stages.

Timing a larvicide application based on monitoring the fly invasion will provide maximum effectiveness. Larvicides must be applied when larvae are susceptible. This is especially true for the insect growth regulator, methoprene. In order for this chemical to be effective, the fourth-instar larva must ingest it, making monitors and an appropriate system of record-keeping essential to time its application.

The location of the larval grazing area must be anticipated. Sciarid larvae tend to move up into the casing from the compost or remain in the casing. Thus, a compost drench prior to casing is not efficacious for an invasion of flies after the casing is in place.

Various predatory mites, *Stratiolaelaps (Hypoaspis) miles* (Berlese), *Geolaelaps (Hypoaspis) aculeifer* (Canestrini), and *Stratiolaelaps scimitus* (Womersley), have demonstrated success against various sciarid species (Castilho et al., 2009).

Larval mortality by biological and biorational products is slower than the rapid organophosphates and the carbamates. Mortality is measured in days as opposed to minutes. Overall effectiveness varies with the product and the timing. Cyromazine and diflubenzuron are more efficacious than the predatory mites, nematodes, microbial pesticides, or insect growth regulators.

11.1.1.4.4 Chemical Control

Chemical insecticides typically target adults in production rooms, work areas, or surfaces where the flies will rest, swarm, and roost. Growers should also treat walls, door jambs, and the plastic cover that is placed over the compost after spawning. Adulticides in the form of aerosols or dusts should be applied when the action threshold is reached.

Resistance to insecticides is a concern. Brewer et al. (1989a, b) in Pennsylvania (USA) and Delaware (USA) have demonstrated resistance of this sciarid to permethrin and dichlorvos, two widely used adulticides. Many insecticides commonly used in the mushroom industry are metabolized by the enzyme system implicated in permethrin and dichlorvos resistance. Adulticides should be used sparingly and alternately with other products of different mode of action to extend the effectiveness of the product.

The use of chemical insecticides can be an important part of gnat control on a farm, but growers should integrate their application with other practices.

11.1.2 Gall Midges, Cecids

Gall midges or cecid flies are small rarely seen flies. They are economic pests of agricultural and food crops, forest trees, and ornamentals.

11.1.2.1 Damage

Cecid larvae (Figure 11.2) feed on the outside of the stipe or at the junction of the stipe and gills of both *Agaricus and Pleurotus* species. Their presence can result in direct yield loss or in a loss of quantity of fresh or processed marketable product.

Most of the literature has not attributed direct yield loss to a cecid infestation. However, White (1990) demonstrated that yield loss can occur when as few as four larvae per square meter are present during spawn-run because of its reproduction potential (see Life History). By mid-cropping 3.5 million larvae per square meter have been observed (Wyatt, 1960).

The actual presence of the orange or white larvae on the stem and gills make the product unmarketable. And, as the larvae migrate from the casing and across the mushroom tissue, their presence induces moisture accumulation and they spread the bacteria that induce browning. Together, the quality loss is substantial.

11.1.2.2 Identification

Gall midges (family Cecidomyiidae) are small, rarely seen flies, about 1.5 mm in length. Several species are associated with commercial mushroom production: *Mycophila speyeri* Barnes, *Heteropeza pygmaea* Winnertz, and *Mycophila barnesi* Edwards (Chung and Snetsinger, 1965). Midge larvae are white (*Heteropeza* spp.) or orange (*Mycophila* spp.); mature larvae are about 2 mm in length. Adult *Mycophila* can be distinquished from *Heteropeza* adults by the former have wing venation and the latter none.

Figure 11.2 Cecid larvae, orange cecid larvae on a mushroom. Credit: Photo graciously provided by Oscar Lahmann. (*See color plate section for the color representation of this figure.)*

When populations are high, their larvae are readily noticed because they wander off the beds and accumulate in heaps on the floor.

11.1.2.3 Life History

Gall midges in mushroom cultivation reproduce by a unique process called paedogenesis. Cecids do not need to mature to the adult stage to reproduce. Instead, a mature (mother) larva will give birth to 10 to 40 daughter larvae without becoming an adult and mating. This can occur in a week or less. Thus, in a few weeks the number of larvae can multiply exponentially. Developmental time is dependent on temperature with the optimum around 240°C. Chung and Snetsinger (1965) observed non-hybrid *Agaricus* brown strains producing twice as many larvae as non-hybrid whites.

Adult development may be triggered by environmental or nutrient stress suggest Chung and Snetsinger (1968). These larvae feed for about 14 days, pupate, and produce adults in 18–21 days. At the time of primordia initiation, there are no mushrooms. So, larvae must feed on mycelia in the casing and on the forming mushrooms. At maturity, the larvae construct pupation chambers of mushroom compost and enter a one-day prepupal stage. After pupation, adults emerge and become active. Cecid development is strongly influenced by temperature. At 7°C, the total generation time is 103 days. At a substrate temperature of around 24°C during the spawn- and case-run production periods, first-generation flies can emerge within 18 days. During later stages of production, when the substrate temperature is allowed to drop to about 19–21°C, the developmental time per generation lengthens to about 21 days.

11.1.2.4 Management Strategies

Gall midges are associated with infested casing material, especially peat. And, they disperse on inadequately sterilized growing surfaces (trays, shelves, netting) and especially on tools, equipment, and workers' shoes and clothing. Any practice that minimizes fly dispersal contributes to gall midge control.

11.1.2.4.1 Cultural Management

Practices similar to those employed for sciarid fly control should be implemented for cecid management.

11.1.2.4.2 Biocontrol/Biorational Control

There is little published research on the evaluation of any product against Cecidomyiidae of commercial mushrooms. White and Czajkowska (2000) determined that there was a sublethal effect of methoprene (*in vitro*) on both *Mycophila* and *Heteropeza* where larval fecundity was reduced and paedogenic life cycle extended, suggesting that this might be helpful in cecid management.

Chemical management. Older literature suggested the use of diazinon or lindane to depress the larval population growth (Hussey et al., 1960). White and Czajkowska (2000) determined that there was a sublethal effect of permethrin (*in vitro*) on both *Mycophila* and *Heteropeza* where larval fecundity was reduced and paedogenic life cycle extended, suggesting as with methoprene that this might be helpful in cecid management.

11.1.3 Phorid Flies

Flies in this family of insects are world-wide and are a pest of mushrooms in many countries. They are commonly known as humpbacked flies or scuttle flies. Six species have been reported

from commercial mushroom facilities in the US (Robinson, 1977). The predominant species in the recent literature is *Megaselia halterata* (Wood).

11.1.3.1 Damage

Megaselia halterata larvae feed at the growing hyphal tips of the mushroom mycelium. This species, unlike *Megaselia nigra* and *agarici* that were pests in the 1940s in the United States, does not consume the sporophores. Thus, direct yield loss correlates to the number of larvae grazing on the mushroom mycelium. More than 12,000 females per square meter of production surface are necessary before significant yield loss occurs (Rinker and Snetsinger, 1984), which is 12 times more than the number required for *Lycoriella ingenua* (syn. *Lycoriella mali*) (Kielbasa and Snetsinger, 1980). Although direct yield loss can be a problem, the greater threat is the transmission of *Lecanicillium fungicola* (syn.*Verticillium fungicola*).

11.1.3.2 Identification

The eggs are about 0.2 by 0.5 mm and lack surface sculpture. Fertile eggs are translucent; whereas, infertile ones are cloudy and opaque. The larvae lack an apparent head capsule and possess posterior respiratory horns, thus differing from sciarid larvae (Figure 11.3); and the first, second, and third instar larvae have cepahlo-pharyngeal skeletons measuring 0.5, 0.8 and 1.14 mm, respectively. The pupae (puparia) are approximately 2 mm in length. Young pupae are whitish with respiratory horns barely visible. Older pupae are yellow-brown with fully developed horns. The outline of the adult fly is visible through the puparium near the time of eclosion. The dark, proctal plates of the male can easily be seen as the pupae near eclosion. The adults of both sexes are small, measure 2–3 mm, lack forked veins and cross veins in the wings, and are easily recognized by their "humpbacked" appearance, laterally flattened hind femora, and quick jerky movements.

11.1.3.3 Life History

Adult females are attracted to actively growing mycelium and oviposit near the hyphal tips. In commercial operations, the mycelium is actively growing about four days after spawning and after casing. Unspawned compost does not support reproduction. Adult females mate in 24 to 48 hours after eclosion, and have a two- to three-day pre-ovipositional period before laying about 50 eggs unlike the sciarid fly, which starts oviposition within 6 hours of eclosion. The average developmental time from egg to adult at 16 and 24°C is 51 and 37 days respectively, with adults surviving 4–8 days.

Figure 11.3 Sciarid larvae with distinctive black head capsule.

Newly emerged and older adult phorid flies readily fly to a suddenly exposed light, especially the shorter wavelengths of blacklight, blacklight blue, or cool white. Outside, flight activity is restricted to the daylight hours. The larvae, pupae, and adults of *M. halterata* are frequently parasitized by an endoparasitic nematode, *Howardula husseyi* Richardson (Figure 11.4), Hesling, and Riding (Tylenchida: Allantonematidae). Parasitism by this nematode does not obviously change the external appearance nor appreciably affect the length of the fly life-cycle; its most significant effect is the reduction of fly fecundity. Laboratory fly populations can be virtually annihilated within five generations by this parasite.

11.1.3.4 Management Strategies

The integrated pest management strategies for control of sciarid flies are effective in managing phorid flies.

11.1.3.4.1 Monitoring

Shorter wavelength lights than those used for sciarids are more effective for phorid flies. The action threshold can be at least five times higher than that for sciarid flies.

11.1.3.4.2 Biological/Biorational Control

Neem-based products along with other plant botanicals will suppress *Megaselia* populations (Erler et al., 2009a,b). The juvenile hormone mimic, methoprene, may show some activity against them (White, 1979). The microbial, *Bacillus thuriengiensis* var. *israelensis* has shown control under field situations (Keil, 1991). The insect growth regulator, diflubenzuron (Scheepmaker et al., 1997), and the entomopathogenic nematodes, *Steinernema feltiae*, and *S. carpocapsae* (Navarro et al., 2014) do not control these phorids.

The natural parasitic nematode populations can be favored by not allowing compost temperatures to exceed 27°C.

11.1.3.4.3 Cultural Practices

Since the phorid is smaller than the sciarid fly, the size of screening must be smaller to prevent fly passage.

11.1.3.4.4 Chemical Control

Generally, phorids are more sensitive to the chemicals registered for sciarids. Since phorids are attracted to the smell of the actively growing mycelium, adulticides should be applied about

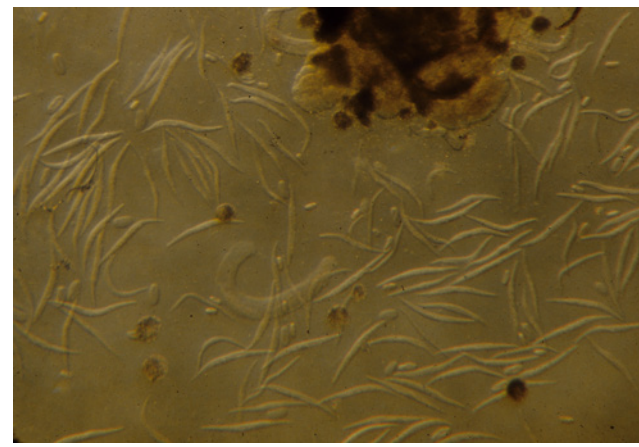

Figure 11.4 *Howardula husseyi*, a macerated female phorid fly showing *Howardula husseyi larve*, gravid female, and eggs.

Figure 11.5 Red pepper mites on the surface of mushrooms.

four days after spawning and/or shortly after casing. Monitor counts will dictate the appropriate timing of the influx of flies.

11.2 Mite Pests

Several groups of mites have been associated with mushroom cultivation. These include: gamasids, pyemotids, tarsonemids, and tyroglyphids. The predominant group familiar to most mushroom growers is the pyemotid or red pepper mites (Figure 11.5).

11.2.1 Pyemotid mites

Red pepper mites, also known as pyemotid or pygmy mites, actually feed on molds (*Trichoderma*, *Monilia*, and *Humicola* spp.). They seem to be found only on production of the commercial button mushroom, where they may cause losses in marketable yield.

11.2.1.1 Damage

Red pepper mites do not cause direct damage to cultivated mushrooms, but their presence often contributes to a loss in marketable yield. They are a nuisance to mushroom harvesters. Importantly, their mere presence indicates other problems (Clift and Terras, 1995). Indirectly, the mites vector diseases, especially *Trichoderma aggressivum*. These mites can carry the *Trichoderma* spores in special areas under their legs (Keil, 1996). And, thus, they can contaminate uninfected areas with *Trichoderma*.

11.2.1.2 Identification

A number of species of pyemotid mites have been identified from mushroom farms (Wicht, 1970). These mites (family Pyemotidae) are tiny, 0.25 mm in length, and yellow-brown.

11.2.1.3 Life History

The mites have a sexual adult stage and a generation time of 4–5 days. Adult females may lay up to 160 eggs over a 5-day period. They do not live on *Agaricus* mycelium but require other

molds for development. Thus, their presence alone suggests the presence of other molds, not necessarily *Trichoderma*, in the compost.

11.2.1.4 Management Strategies

Proper compost preparation and its pasteurization will minimize weed molds and eliminate the food source for red pepper mites, thereby eliminating red pepper mite populations. Red pepper mites can survive pasteurization temperatures on compost drier than 68% (Clift and Terras, 1995). The casing surface at the post-crop heat treatment needs to be moist as well.

These mites tend to congregate on top of the carpophores and can be seen by shining a light across the pre-harvested mushrooms. They have the appearance of "red pepper" on the mushroom surface, hence, the common name "red pepper mites." This behavior enables dispersal by flies or harvesters' clothing. Water splash during the irrigation process moves mites to other shelves and to the floor. Attention to fly management, common equipment moved between production rooms, harvesters' clothing and shoes, picking baskets, and sales containers (a.k.a. punnets) is required to minimize spread of the mites and molds.

11.2.1.5 Chemical Management

Miticides are not registered for control. Some producers have found it "helpful" to reduce the number of mites on the mushrooms for sales by first applying a registered fungicide to the casing followed a few days later by a registered chemical insecticide drench.

11.2.2 Gamasid Mites

This group of mites is predatory. They will feed on other mites, fly eggs, and larvae and nematodes. In general, these mites can be recognized as a group because they are fast moving on the compost or casing surface. Fletcher and Gaze (2008) note three species: *Parasitus fimetorum*, *Digamasellus fallax*, and *Arctoseius cetratus.*

This mite group is an indicator of other crop situations. They will enter the crop attached to flies. And, they will reproduce on other living things in the compost or casing. Since they move about the substrate seeking a prey, they will spread other problems throughout the crop.

Control of this group is through the management of situations or conditions that permit their entrance into the crop and survival.

11.2.3 Other Mites Associated with Mushroom Cultivation

These mites include *Tyrophagus* spp., *Caloglyphus* spp., *Histiostoma* spp., and *Linopodes antennaepes* (Fletcher and Gaze, 2008; Snetsinger, 1972). These mites do not feed on *Agaricus* mycelium but other fungi present in the crop. Their presence indicates problems with poorly made compost and/or inadequate pasteurization and conditioning.

The *Tyrophagus* spp. and *Linopodes* spp. have been associated with pitting and browning of the stipe. However, this may be a secondary situation and not the primary cause for the bacterial problem.

Control of this group is through the management of situations or conditions that permit their entrance into the crop and survival.

11.3 Nematode Pests

Mushrooms nematodes can be grouped into at least two broad categories: beneficial and harmful. Mushroom growers have typically thought of nematodes as being harmful to the crop.

Parasitic (mycophagous) and saprophytic nematodes are associated with commercial mushroom yield loss. The occurrence of mycophagous nematodes in mushroom culture is rare under modern mushroom farming techniques. Saprophytic nematodes are common. Generally, their overall economic impact on production is minimal; however, under heavy populations significant individual crop reduction is experienced.

Beneficial nematodes, on the other hand, control insect populations. They may be either natural to the population or introduced to the mushroom crop to control mushroom pests.

11.3.1 Saprophytic Nematodes

There is inconsistency as to the correlation of nematode populations and mushroom yield reductions. Nevertheless, all commercial mushroom operations attempt to minimize these free-living nematode populations.

11.3.1.1 Symptoms and Signs

In single-zone systems black necrotic areas may be visible on the spawned compost surface prior to laying of the casing. These spots may show some colonized compost but the mycelium will be fragmented and the compost wet. These areas will not be re-colonized by the mushroom mycelium. The surrounding colonized compost will degenerate, and the nematodes will migrate into the casing layer. With careful observation and a bright light, the nematodes can be recognized as they flicker (reflecting the light) on the compost straws.

Often the casing is well colonized by the mycelium but after ruffling or scratching, the mycelium does not re-knit well in spots or the whole shelf. Sometimes the casing will initially be slow to be colonized by the mycelium. The mycelium is fragmented and the casing does not hold together well. As a consequence, yield is reduced (Kaufman et al., 1984). Sometimes growers confuse the die-back symptom of virus disease with nematode problems. As in the compost, the flickering nematodes may be observed on the casing surface with a bright light.

The whiteness of the mushroom may be negatively affected. Bacteria on which the nematodes feed reproduce well in the moist environment created by the nematodes. The nematodes and their associated bacteria lower the quality of the fresh mushrooms (Grewal, 1991).

Sometimes brown fungal colonies develop on the compost or casing. This fungus, *Arthrobotrys* spp., is a parasite of nematodes. Its presence is an indicator of high nematode populations (Fletcher and Gaze, 2008).

11.3.1.2 Causal Agents

The majority of saprophytic nematodes recovered from mushroom casing samples belong to the genera, *Acrobeloides*, *Rhabditis, Choriorhabditis,* and *Caenorhabditis* in the order Rhabditida. They are all bacteria feeders, characterized by having three or six lips fused or replaced by other structures. Their stoma lack a stylet and their cuticle is annulated or smooth. Amphids are inconspicuous and the esophagus has a terminal bulb. The tail of the male usually possesses a bursa supported by rays. There are no caudal glands.

11.3.1.3 Disease Cycle and Epidemiology

Saprophytic nematodes are common inhabitants of compost and casing mixtures. Under optimum conditions, 50–100-fold increase each week is possible. Under slow drying conditions, especially during the pre-pasteurization phase of compost, some nematodes can form resistant stages which can enable them to survive pasteurization temperatures. Insects, equipment, workers, and irrigation of the casing can disperse the nematodes. In older farms with wooden ceilings, nematodes can reproduce in the wet insulation and drop onto the compost through the condensation on the ceiling.

11.3.1.4 Management Strategies

Good sanitation and hygiene practices reduce the spread of nematodes on the farm. Thorough post-crop pasteurization and cleaning of the production room, netting, and equipment will reduce carry over from one crop to another. Farms with wooden shelving or trays need to do an especially thorough post-crop heat treatment since the nematodes may be located in crevices of the wooden boards and survive the post-crop heat treatment.

Maintenance of proper temperatures during pasteurization of compost is critical to reduce the nematode threat. If the surface of the compost becomes dry during the pre-pasteurization phase, resistant stages of the nematodes may survive the pasteurization temperatures. Changes in the length of the pre-pasteurization period, air flows, and humidity control may be necessary.

Insects are excellent vectors of nematodes (Rinker and Bloom, 1983). A good integrated pest management program is required to reduce this additional impact of flies on a mushroom crop.

The casing material can be a source of nematodes. Although peat may have different levels of nematodes, generally, pre-packaged peat is no problem in mushroom production if it is properly handled. Once opened, the peat should be mixed in a cleaned area with cleaned equipment and used within 24 hours. However, if the bags become broken and the peat becomes wet, nematodes will multiply. These bags should be discarded away from the farm.

Once nematodes are noticed on the compost or casing the best control is to reduce their spread in the room and on the farm and to determine the source or cause of infestation. Tools and equipment should be sanitized between shelves during the spawning operation. Ruffling or scratching should be avoided where nematodes are visible on the casing. There are no registered chemical controls.

11.3.2 Parasitic Nematodes

Parasitic or mycophagous nematodes were serious pests of commercial mushroom production. Extensive sampling of commercial mushroom facilities in Canada and the USA over the past 50 years has not revealed the presence of these nematodes. However, they remain problematic in other regions of the world (Katyal et al., 2007). Hussey et al. (1969), and Goodey (1960) provide good discussion of this group.

11.3.2.1 Causal Agents

Mycophagous nematodes are in several genera: *Ditylenchus myceliophagus* and *Aphelenchoides composticola.* These nematodes have mouth parts that can penetrate the *Agaricus* mycelium and feed exclusively on the mushroom mycelium, destroying it.

11.3.2.2 Symptoms and Signs

The symptoms and signs of mycophagous nematodes are similar to that of the saprophytic ones. The fine strands of the mycelium are destroyed and the casing/compost becomes soggy and smells with little or no production in the area.

11.3.2.3 Disease Cycle and Epidemiology

These mycophagous nematodes may be found in soil and peat. One female can produce up to 500 eggs. They can increase rapidly and reproduce as much as 25,000 times in one week.

As the food source disappears the nematodes group together in clumps or swarms. As with the saprophytic ones, a light on the compost or casing surface will reveal their presence. This behavior permits "easy" access to flies that can transport them to other locations. These nematodes can survive for six weeks without feeding and in a desiccated state for upwards of three years. Poorly post-crop heat treated spent compost could be a good source of these nematodes.

11.3.2.4 Management Strategies

These mycophagous nematodes may be found in soil and peat. Avoiding incorporation of soil into compost, avoiding runoff from soil onto the compost or compost ingredients, or preparation on soil will minimize the risk from infection.

Maintaining adequate uniform phase II pasteurization temperature will assist in elimination of this nematode. If the compost dries during warm-up to pasteurization, then some nematodes may go into a resistant stage and infest the crop at spawning.

Once an infection site is identified, it is quite important to maintain vigilant control of flies. Covering the infected area with plastic will minimize movement by personnel, insects, or irrigation. Adequate management of temperatures during post-crop heat treatment, disposal of the spent material away from the farm, a thorough cleaning of the room prior to reuse and a heat treatment of the cleaned empty room before fresh substrate is added are critical.

No chemicals are registered for control although some literature suggests azadirachtin or diflubenzuron (Gahukar, 2014) and the fungicides benomyl and thiabendiazole (McLeod and Klair, 1978) may reduce population growth of *Aphelenchoides*.

11.3.3 Beneficial Nematodes

Beneficial nematodes are new to the thought processes of mushroom farmers. Beneficial nematodes can kill the host directly, reduce its ability to produce offspring, or be a carrier of disease to the insect. These nematodes fit well into biological control strategies for insects.

11.3.3.1 Endoparasitic Nematodes

The phorid fly (*Megaselia halterata*) has within this insect's population a naturally occurring nematode, *Howardula husseyi*. The nematode enters the fly larva and reproduces within the fly. As the nematodes develop inside the fly, they consume the fly's reproductive system. Thus, an infected female produces few, if any, offspring. When she attempts to lay her eggs, the nematodes are released back into the compost or casing, ready to infect other phorid larvae (Figure 11.6). Commercialization of this nematode for biological control has not been successful.

11.3.3.2 Entomopathogenic Nematodes

Another more successful commercial nematode product for mushroom fly control has been the development of nematodes that enter the fly larva and release infective bacteria that kill the

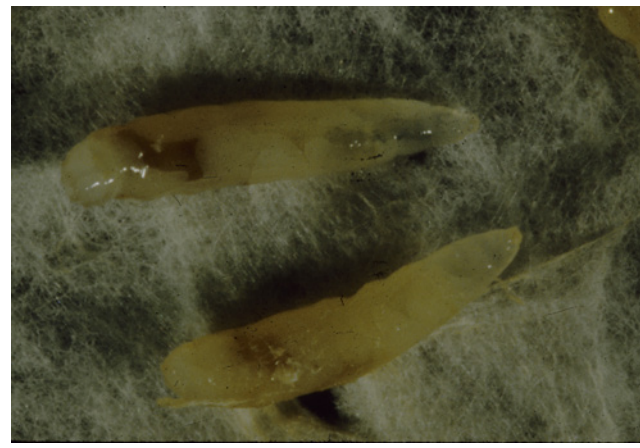

Figure 11.6 Phorid larvae. (*See color plate section for the color representation of this figure.*)

fly larva. These nematodes either bore through the body of the insect or go through natural openings in the insect larva. Once the infective-nematode enters the fly larva, it releases bacteria that it carries in its digestive system. As the fly larva dies the nematode uses the cadaver to reproduce more young.

One of the more common nematode species for mushroom fly control is *Steinernema feltiae*. It is one of most effective species against the sciarid fly, *Lycoriella ingenua* (syn. *Lycoriella mali*) with 70% average control at the farm level. However, no entomopathogenic nematode species has been reported to control the phorid fly. The nematodes are typically applied to the casing material in the irrigation water with the timing of application important to maximize control. These nematodes prefer to attack large fly larvae or young pupae, about 12–16 days from egg laying. This necessitates appropriate monitoring of invading adult populations. The nematodes may reduce the mycelial growth on the casing surface with a slightly weaker mycelial growth in the casing at pinning time when commercially recommended rates are applied at casing time. Yield at commercial rates are not affected; first break may be delayed up to one day (Rinker et al., 1995, 1997).

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12

Mushroom Diseases and Control

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12.1 Introduction

Present-day mushroom cultivation is carried out in mushroom growing farms that facilitate the practice of IPM programs. The facilities are characterized by the ability to control environmental conditions by means of heating and cooling systems, air circulation systems, and so on. However, even under such controlled conditions, mushroom crops are susceptible to a variety of viral, bacterial, and fungal diseases that may affect mushroom yield and quality.

Phytosanitary products, especially fungicides, can help control some mushroom diseases, although the use of many products is strictly limited by official regulations or by the similarity between pathogen and host. The use of any fungicide may, therefore, cause a drop in yield, so it is necessary to use products with a contrasted specificity, that is, fungicides that are toxic to the pathogen and do not damage the development of the mushrooms. Currently, chemicals used alongside optimized hygiene strategies based on the detection of pathogens at a very early stage of the cultivation process allow infections to be partially controlled, although recent studies on natural compounds, especially essential oils, point to a new and particularly promising way of disease control.

12.2 Fungal Diseases

12.2.1 Dry Bubble

Dry bubble, whose causal agent is *Lecanicillium fungicola* (syn. *Verticillium fungicola*), is one of the most common and serious fungal diseases of mushroom (*Agaricus bisporus*) crops. *L. fungicola* is a hyphomycete parasite of *A. bisporus* carpophores but not of the vegetative mycelium. On agar-malt medium, it forms rounded colonies with a regular edge and radiating. It is powdery or cottony in appearance, with a white to pale cream color. The conidiophores are erect, with 1–10 whorls on the main axis, and 2–9 conidiogenous cells per whorl. Conidia range from cylindrical to ellipsoid, also oval, with an obtuse apex, are hyaline, single-celled, with an average size of 7.5 × 2.5 μm. The optimal temperature is 20°C.

There are several aspects of the biology of *L. fungicola* that affect the evolution of the disease. The most important is the presence of spherical masses (clusters) of conidia (up to 64) covered by sticky mucilage. The mucilage surrounding them facilitates adherence to any surface that

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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comes into contact with them (tools, clothes, pickers, diptera, etc.), which is why *L. fungicola* spreads so easily.

The primary source of *L. fungicola* in mushroom crops is the casing material and, especially, peat. The air in ventilation systems can play an important role in disseminating it inside the mushroom farm or even transporting it to other installations since conidia can adhere to specks of dust or other debris.

Two groups of symptoms are associated with infection by *L. fungicola*. These are of varied appearance and depend on the stage of development of the fruiting bodies in which infection takes place. When the mushrooms are infected at an early stage of development (the primordial stage,) small spherical masses of tissue appear in which there is no difference between stipe, cap, or gills. Generally, the infection requires between 10 and 14 days of incubation before these symptoms manifest themselves. In the beginning, these masses are white, like the mushrooms. Afterwards they acquire grayish tones, which indicates that sporulation of the pathogen has begun. These spherical masses grow up to 1–2 cm in diameter, although if not disposed of in time they may exceed 4–5 cm (Figure 12.1).

If the mushrooms are affected in a more advanced stage of their development, when stipe and pileus have already become differentiated, they are often imperfectly formed. The stalk arches, causing inclination of the cap. There is also swelling in the basal part of the stipe. In addition, the pileus is abnormally small, deformed, with warts or bumps, and inclined. These sporophores are covered by a thin whitish-grayish mycelial proliferation, and, although discolored, they do not rot, so they do not produce off-smells; instead, they look parched and have a leathery texture. When the aggression takes place on the stalk, the infected cells stop growing, while the non-infected ones continue to grow, so the stipe curves, causing a rupture of the external tissues (Figure 12.2). The final result is that the stipe looks as if it had been peeled from top to bottom; one side of the cap is often affected, and protuberances develop which resemble a hare's lip. If the mushrooms open, the pathogen is able to parasitize the gills of the cap. Normally this does not happen, since the fruiting bodies are collected before the internal veil that covers the gills breaks.

When the infected carpophores are almost completely differentiated, they become covered with spots, which vary from chestnut brown to matt gray. The brown color corresponds to an incipient infection, while gray reflects greater progression in the mycosis infection and is due to sporulation of the pathogen in the cap. Necrotic lesions also appear due to the penetration

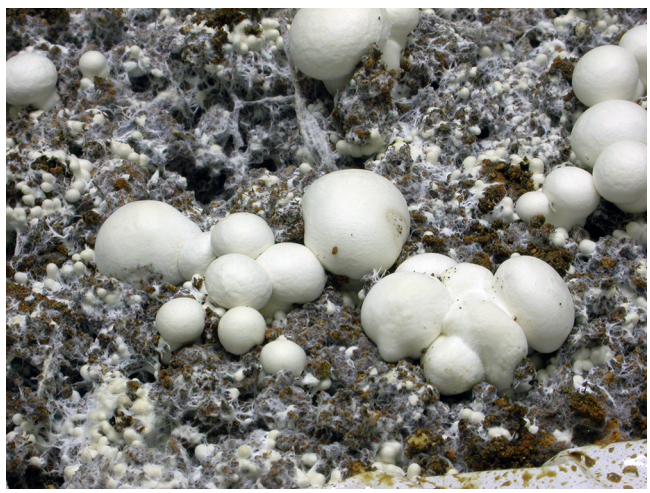

Figure 12.1 Undifferentiated masses of mushroom tissue as a result of infection by *Lecanicillium fungicola*.

Figure 12.2 Imperfectly formed mushroom with distorted stalk caused by *Lecanicillium fungicola*.

Figure 12.3 Cap spotting of *Agaricus bisporus* caused by *Lecanicillium fungicola*.

of the mycoparasite into the internal tissues of the cap. This symptom generally appears after approximately 7 days of incubation.

In general, spots are irregularly shaped and can coalesce, occupying a large part of the surface of the cap. In the case of the darker spots, a whitish-yellowish halo can be seen surrounding them. If the environment remains wet, some whitish-grayish mold can appear, corresponding to the vegetative mycelium of the mycoparasite.

As regards the critical period for the crop, the casing is particularly vulnerable when the inoculum is transported by air. The disease is more severe when the inoculum level in the surface of the casing is high and becomes evident at the first flush. If the concentration of inoculum is low, the chances that the spores get close to the developing mushrooms are low, and the symptoms may not appear until the second or third flush.

Only visual examination of the crop surface allows dry bubble symptoms to be detected. However, if the symptom that appears is a cap spotting (Figure 12.3), it is necessary to confirm the presence of *Lecanicillium fungicola* in petri dishes with medium (PDA, malt extract agar), because it may be confused with spots caused by other pathogens such as *Trichoderma aggressivum*, *Cladobotryum mycophilum*, or *Pseudomonas tolaasii*.

12.2.1.1 Control

- Any activity associated with mushroom crop should always be carried out on the newer crops before the older ones.

- Implement measures that favor the exclusion of diptera from the mushroom farm.
- Keep casing materials in places where they are not liable to be contaminated.
- Remove all affected mushrooms before watering or harvesting. Pay special attention to watering and to the handling of tools on the mushroom farm.
- If not removed quickly, dry bubbles can be covered with salt and have a plastic cup placed over them.
- Do not transport nozzles or hoses from old to new mushroom growing crops without previously disinfecting them.
- Use new or disinfected collector boxes that do not come from contaminated crops.
- Do not lengthen the crop cycle unnecessarily.
- Thoroughly clean and disinfect the mushroom farm once the crop cycle is complete.
- Prochloraz and chlorothalonil are the most effective fungicides in the control of dry bubble.

12.2.2 Wet Bubble

Wet bubble is caused by *Mycogone perniciosa*, which is characterized by the presence of cream to pale leather colored mycelium on a PDA medium, while the reverse is less colored. With the passing of time, the predominant color is leather due to the formation of chlamydospores. It has conidiophores of two kinds: the first are more or less erect, branched, septated, hyaline, 150–450 × 4–5 μm, with 2–4 whorls, and occasionally secondary whorls. Whorls with 3–5 conidiogenous cells of 20–40 × 3–4 μm, thinning to 1 μm at the apex. Terminal conidia are solitary, thin-walled, hyaline, of ellipsoid cylindrical shape, slightly pointed at both ends or with a rounded apex and the other pointed; unicellular or bicellular, 11–15 (22) × 2.5–4 μm. The lowest conidiophores have irregularly distributed chlamydospores, either terminal or lateral. The chlamydospores are bicellular: the upper cell has a thicker wall, covered with small warts or spines, rounded, slightly pigmented, 13–30 × 16–31 μm; lower cell has a thin wall, cup shaped, hyaline, smooth, 8–15 × 8–18 μm.

Usually, the mycoparasite produces more chlamydospores than conidia in an artificial medium, while in the infected fruit bodies, the contrary occurs. Conidia germinate freely on various substrates, although chlamydospores can survive dormant and germinate only when they are activated by factors arising from the vegetative mycelium of mushrooms and the tissues of basidiomas.

Mycogone perniciosa parasitizes the carpophores of *A. bisporus* but not the vegetative mycelium. The main source of inoculum is the casing material, while compost is not cited as a major source. The pathogens are spread by splashing of water, flies, and operators (tools, hands, clothes, etc.). It is therefore necessary to pay strict attention to hygiene, especially to the casing materials, and eliminate the primary sources of inoculum. Conidia can also be transported by air.

Infection at a very early stage of mushroom development gives rise to the most characteristic symptom of the wet bubble: the formation of deformed tissue masses of the carpophore, which can reach 10 cm in diameter (Figure 12.4). They are initially white and fluffy, and as they grow, they acquire a brown color. Later they secrete amber liquid drops containing bacteria and spores. They eventually rot, releasing an unpleasant smell.

If the attack takes place when the mushrooms are almost developed, the base of the stalk and a sector of the cap may appear deformed and covered in white mycelium (Figure 12.5). In short, the later the infection occurs during growth of the mushroom, the lesser the resulting distortion. Wet bubble shows symptoms that can sometimes be confused with those produced by dry bubble. The main differences are that the pathogenic fungus behind wet bubble causes the appearance of a dense, velvety, and white mycelium, rotting of the inside tissue of

Figure 12.4 Undifferentiated tissue mass of *Agaricus bisporus* affected by *Mycogone perniciosa*.

Figure 12.5 *Mycogone perniciosa* on the surface of developed mushrooms. Amber droplets of liquid can be seen on the diseased mushrooms. (*See color plate section for the color representation of this figure.)*

the mushroom, and exudation of drops of foul smelling amber liquid, accompanied by the massive invasion of bacteria.

In general, wet bubble manifests itself in the first and second flushes, probably because the casing material is the main source of inoculum. Also critical are soil movements near the mushroom farms, or the emptying of the growing rooms, which may be contaminated.

12.2.2.1 Control

- The necessary cultivation practices should always be carried out on the newer crops before the older ones.
- Implement measures that favor the exclusion of diptera from the mushroom farm.
- Keep casing materials in places where they are not liable to be contaminated.
- Eliminate all affected mushrooms before watering or harvesting. Pay careful attention to watering and handling tools in the growing room.
- Do not prolong the crop cycle unnecessarily.
- Carry out deep cleaning and disinfection of the growing rooms once the crop cycle is completed.
- Prochloraz and chlorothalonil are the most effective fungicides in the control of wet bubble.

12.2.3 Cobweb

In recent years, cobweb has become a common disease in mushroom crops, coinciding with changes in farming techniques, particularly in the type of casing material used and in irrigation.

One of the pathogenic fungi that causes this disease in mushroom crops is *Cladobotryum dendroides*, which is characterized by the presence of phialides with an irregular apex and large conidia (20–30 μm in length) with 3–4 cells (2–3 septa). The mycelium is initially white, and subsequently acquires reddish tones while aging. Recently, the disease has also been associated with the species *Cladobotryum mycophilum*, which is characterized by the presence of conidia with only two cells (a septum) and simple and regular apex of the phialide. In addition, the colonies produce a characteristic smell of camphor.

Common environmental conditions in mushroom crops may promote the development of the pathogen. In fact, germination of the conidia and subsequent growth of the pathogen is favored by high humidity of the substrates or the fruit bodies and an optimum temperature of 25°C. When water does not evaporate properly from the surface of culture or the carpophores, cobweb may become abundant.

The main sources of inoculum may be fragments of mycelium transported by air, or contaminated casing material. The main means of dispersion of conidia is air, and even if the affected area is covered with salt, there may be a significant dispersion of spores. Also, water splashes during irrigation may contribute to dispersion. Mushroom flies and pickers seem to be less important for dispersion, since the spores are not sticky.

The first symptoms of the disease usually appear between the second and third flushes, although they can sometimes appear during the first flush. Problems begin with small circular, more or less regular colonies of mycelium on the layer of casing material or fruit bodies. As the mycelium extends outward, it affects nearby mushrooms. The infection presents a fine white mycelium, resembling a spider's web, which rapidly develops. After a few days, the cottony mycelium becomes denser and presents an abundant sporulation of conidia. The mycelium can quickly trap dead primordia, stipes, and fruiting bodies (Figure 12.6). The affected carpophores, in time, become brown or black, and eventually rot. Normally the grayish white mycelium acquires yellow and pinkish hues in colonies of a certain age, due to the secretion of the pigment aurofusarin.

Another manifestation of the disease is the appearance of spots on the carpophores. Two types of cap spotting may be observed: brown dark spots with a poorly defined border, and light brown to gray spots which occupy most of the surface of the mushroom (Figure 12.7). The

Figure 12.6 Cobweb mycelium (*Cladobotryum mycophilum*) on the surface of the casing and attacking mushrooms (*Agaricus bisporus*). (*See color plate section for the color representation of this figure.)*

Figure 12.7 Cobweb mycelium (*Cladobotryum mycophilum*) causing cap spotting.

dark brown spots can be confused with those produced by other pathogens such as *Trichoderma* spp. or *Lecanicillium fungicola*.

The critical period is after casing. From this moment onwards, it is very important to correctly control the environmental conditions in order to promote continuous evaporation. Watering is also a critical moment, especially when there are already colonies of cobweb on the casing material.

12.2.3.1 Control

Compliance with strict hygiene and environmental control measures is the best way to fight disease.

- Areas of the surface of the casing material or carpophores that are affected should be treated with great care in order not to facilitate the dispersion of conidia. It is recommended that damp paper be placed over the affected area, in such a way that the edges extend beyond the edges of the cobweb colony by several centimeters.
- Do not water or manipulate the areas that are affected by the cobweb.
- Avoid long periods of high humidity on the carpophores and ensure good evaporation of the compost.
- At the end of the crop cycle, perform a cooking-out step on the crops where this can be done.
- Pay special attention to disinfection of clothes and footwear of the staff in critical areas.
- Maximize control of mites and diptera on the mushroom farm.
- Do not use returnable containers without suitable cleaning and disinfection.
- All necessary cropping practices should be carried out on the newer crops first, before the older ones.
- Prochloraz and metrafenone are the most effective fungicides in the control of cobweb.

12.2.4 *Trichoderma* Diseases

There are several species of *Trichoderma* (*T. harzianum*, *T. atroviride*, *T. koningii*, *T. aggressivum*, *f. aggressivum*, and *f. europaeum*, *T. viride*) that cause damage to mushroom growing crops. The disease is characterized by the presence of colonies of rapidly growing dense white mycelium on the casing material or the compost, which changes to green after intense sporulation.

Figure 12.8 Brown spots on a mushroom cap, caused by *Trichoderma aggressivum*.

Trichoderma species are characterized by the production of a large number of conidia which vary in their shades of green. They form into chains of sticky conidia which can be easily scattered by the movement of air when they are on debris, or by insects and mites, particularly pygmephorid mites, or by workers, containers, and so on. Their optimal temperature varies from one species to another, from 22 to 28°C. Some species of *Trichoderma* grow particularly well at pHs lower than 6, especially if the nitrogen level is low. Thus, a C/N ratio of 22–23 favors the growth of *Trichoderma* in compost.

Currently, the most harmful species is *T. aggressivum*, which is characterized by its rapid growth rate (more than 1 mm per hour at 28°C on agar). The form *T. aggressivum f. aggressivum (Th4)* occurs in North America, while *T. aggressivum f. europaeum* is found in Europe *(Th2)*.

The carpophores with spots of *Trichoderma* on the cap are often found at the bottom of shelves or on the compost and on the sides of containers. These spots on the caps develop when there is abundance of inoculum and the mushrooms remain moist for periods of at least 2 hours. It is common for the spores to be transferred through water drops (Figure 12.8).

The emergence of *Trichoderma* on the compost could be due to contamination during spawning. *Trichoderma* grows well on carbohydrates, and in this sense, seed grain is an important source of food, and is very vulnerable. Once installed on the compost, the pathogen is able to colonize large areas, since it is favored by the distribution of the seed grains in the compost mass. Mushroom farm systems that use compost blocks wrapped in polyethylene seem to be particularly vulnerable due to the condensation of water inside the polyethylene, which not only provides an ideal environment for germination of the spores, but also helps to distribute them. Also, using compost blocks involves transportation from the composting factory to the mushroom farm, and the temperature of the compost may not be properly controlled during transport. If the temperature reaches close to the optimum for *Trichoderma* (28°C) it increases the possibility of developing the pathogen. There is also evidence that the copious addition of supplements containing carbohydrates at the time of spawning may favor the development of *Trichoderma*. By contrast, a compost completely colonized by the *Agaricus* mycelium is not affected by the pathogen.

T. koningii causes spots of brown to gray color in the caps of mushrooms, with no clearly defined outlines. These stains are usually small, of no more than 5 mm in diameter, and are often numerous. *T. harzianum* causes larger spots, dark brown, and with a diffuse limit. Sometimes severely affected mushrooms show dry rot in the area occupied by these spots.

The most harmful form of *Trichoderma* is that associated with colonization of the compost. The species involved in this symptom is *T. aggressivum.* The disease frequently appears when a dense white mycelium can be seen, sometimes on the surface of the compost, through the sides

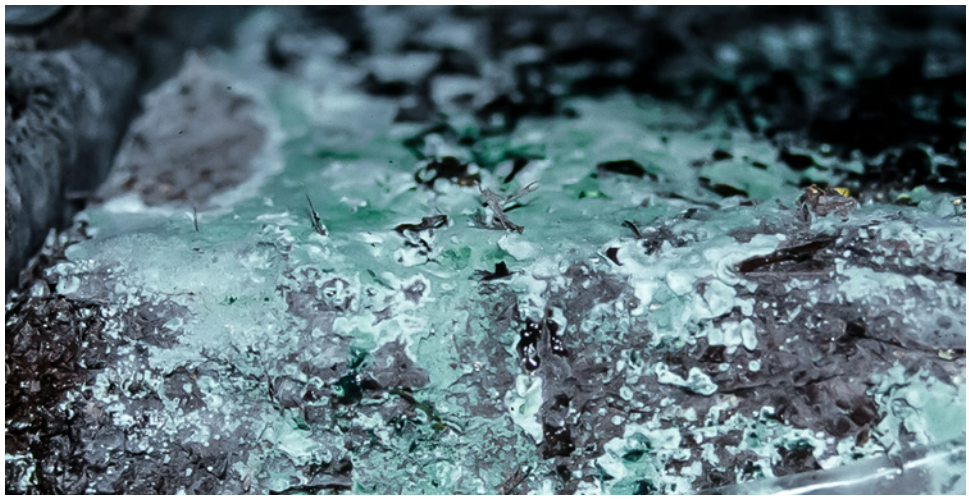

Figure 12.9 *Trichoderma aggressivum* colonizing compost. (*See color plate section for the color representation of this figure.)*

of the bags or packages, or through the mesh of the shelves (Figure 12.9). *T. aggressivum* may appear on the surface of the casing, usually during the first flush. It turns dark green once the spores are formed (Figure 12.10). When evident on compost it causes a considerable reduction in the crop, and quality can also be affected, as there may be spots on the carpophores. Mites (*Bakerdania mesembrinae*) are frequently associated with the presence of *Trichoderma*, since they feed on the fungus. Therefore, the first symptom is often the appearance of these mites.

Critical periods are the time of spawning and packaging of the compost, when it is necessary to take extra precautions. Compost temperature is an important factor in the evolution of the infection, as an excessively high temperature (28°C) may cause the mycelium of *T. aggressivum* to grow 10 times faster than that of the mushroom. Therefore, the stage of colonization of the substrate is also critical, as there must be a good control of the temperature.

Figure 12.10 *Trichoderma* mold colonizing casing.

The presence of *Trichoderma* in the compost can be detected by placing a small amount of compost in petri dishes with PDA medium. After 5–6 days at room temperature, the presence of the pathogenic fungus can be confirmed or ruled out. The same can be done with the casing material, although it is not a very definitive method of detection. Subsequently, visual inspection of these substrates can also confirm the presence of *Trichoderma*. When the symptom that appears is a spotting cap, it is necessary to confirm the presence of the pathogenic fungus in petri dishes with culture medium (PDA, malt extract agar), because it can be confused with spots caused by other pathogens such as *Lecanicillium fungicola*, *Cladobotryum mycophilum*, or *Pseudomonas tolaasii.*

The disease can be followed by counting and locating the spots of *Trichoderma* detected in a mushroom crop cycle. This should reveal certain patterns of behavior that will allow us to assess whether infection is due to health problems in the spawning equipment, to the presence of diptera, or to dust entering the mushroom crop facilities. It is also of interest to know when the disease manifests itself, as if it is detected before the end of the stage of incubation, it is a much more serious problem than if only a few spots of *Trichoderma* are detected during harvest.

12.2.4.1 Control

The strict application of hygienic measures is required when the presence of *Trichoderma* is detected in the compost, or in those mushroom farms that have previously suffered the disease. The detection of a few spots in the first flush also means the enforcement of strict hygienic measures is necessary.

- Ensure good mixing of base materials during phase I of composting and avoid the appearance of anaerobiosis zones.
- Ensure that the whole mass of compost receives good pasteurization. There should be a good flow of air through the whole mass, since *Trichoderma* colonizes the compost much more easily when it has had anaerobiosis problems.
- Place air filters in phase II rooms, areas of spawning, and incubation rooms.
- Prevent the final compost exceeding a humidity level of 70%.
- Ensure that the levels of ammonia during phase II are above 450 ppm.
- The packaging area must be closed and equipped with positive pressure filtered air. These areas should be cleaned and disinfected at least once a week.
- Spawn storage chambers should be washed and disinfected regularly.
- The spawning equipment must be cleaned and disinfected at the end of the day.
- Vehicles should not be allowed in the spawning and packaging areas.
- The packing machinery should be covered at night with a sheet of plastic which must be renewed every week.
- Pay special attention to disinfection of clothes and footwear of the staff in these critical areas.
- Avoid unnecessary operations during the stage of compost incubation.
- Avoid temperatures of over 27–28°C during the incubation phase.
- Maximize control of mites and diptera in the mushroom farm.
- Keep the surface of the compost covered during colonization.
- Do not use returnable containers without suitable cleaning and disinfection.
- The necessary cultivation practices should always be carried out on the newer crops before the older ones.
- Keep casing materials in places where they are not liable to be contaminated.
- The most effective way to control cap spotting is to avoid humid conditions and poor evaporation of the carpophores when they develop.

- Do not allow the carpophores to remain moist during periods of 2 hours or more.
- Remove the carpophores that fall between bags.
- Pay careful attention to watering and handling of the tools in the mushroom farm.
- Do not lengthen the crop cycle unnecessarily.
- Cook out the affected mushroom growing crops whenever possible.
- Perform deep cleaning and disinfection of mushroom buildings once the crop cycle is completed.

12.2.5 False Truffle

Diehliomyces microsporus form cream to reddish brown, subspherical cerebriform ascocarps, of 2–3 cm diameter. Often, these ascocarps come together forming groups of considerable dimensions on the surface of casing material (Figure 12.11). Inside the fruiting bodies there is a layer that is occupied by numerous asci and sterile hyphae. The asci, which are oval in shape, often contain eight ascospores. These spores are almost spherical and measure an average 6 × 5 µm. *D. microsporus* spores ripen 3–6 weeks after the formation of the fruiting bodies. Once the spores are released, the ascocarps disintegrate into a brown mass of powdery appearance.

Ascospores often germinate when the mycelium of the *Agaricus* is beginning to develop, since germination is stimulated by the presence of the actively growing mushroom mycelium. To germinate, the spores need high temperatures of more than 28°C. After germination, the mycelium develops well at a lower temperature, although ceases to grow below 15–16°C, which is why it is unusual for *D. microsporus* to appear during winter. By contrast, it often appears during summer when it is difficult to maintain the temperature lower than 20°C inside the mushroom farm.

The first symptom that can point to the presence of false truffle is the appearance of a dense yellowish-white mycelium in the compost mass at the bottom. This mycelium looks thicker than the mushroom mycelium and is grayish white, although it is initially difficult to differentiate between the two (Figure 12.12). On other occasions, prior to the appearance of this yellowish mycelium in the mass of compost, it has been observed covering the casing material as a cottony white mycelium growing in some parts of the packed substrate. This mycelium evolves to form fruiting bodies with the appearance of grayish white peeled walnuts. This appearance

Figure 12.11 Mycelium and ascocarps of *Diehliomyces microsporus* on the casing surface. (*See color plate section for the color representation of this figure.)*

Figure 12.12 Dense mycelium of *Diehliomyces microsporus* on the compost.

is responsible for the other name for this pathology: calves' brains. The fruiting bodies can coalesce to form large masses that occupy most of the substrate package, and may be found in both the compost and the casing material, or at the interface of both. When these ascocarps mature, they acquire a rusty color.

The presence of false truffle inhibits growth of the mushroom mycelium on the compost, which sometimes disappears completely, with the compost being soaked, abnormally black, and causing a sharp decline in production. Mushrooms that grow on the edge of the affected area acquire a yellowish color and die before reaching an acceptable size. Significant losses in harvest may occur when the crop is affected before or during incubation, with yield reductions of up to 75%. This pathogen has been detected in both the pre-fructification phase and harvesting phase.

The main source of contamination is soil, which may be found in unpaved composting areas or in bales of straw mixed with soil during harvesting, transport, or storage. The most critical period is incubation, especially at the beginning, when the temperature of the compost is higher. Commercial varieties of *Agaricus bitorquis* are generally more susceptible to false truffle than varieties of *A. bisporus,* mainly due to the higher cultivation temperature used for *A. bitorquis.*

12.2.5.1 Control

- Use suitable temperatures for phase II of composting.
- Ensure at least 450 ppm of free ammonia, measured 3 hours after phase II reaches its maximum temperature.
- Prevent contamination of the soil in the composting area. Clean the work area adequately. Use cemented compost areas.
- Use straw that is free of soil.
- Avoid temperatures above 30°C during incubation of the mushroom mycelium.
- When possible, cook out at the end of the crop cycle.
- Properly disinfect the mushroom farm between crop cycles whenever it is not possible to cook out.
- Cultivation practices should be carried out on the newer crops before the older ones.
- Keep casing material in places where they are not liable to be contaminated.
- Pay careful attention to irrigation and the handling of tools in the mushroom farm.
- Cover any false truffle ascocarps with damp paper.
- Do not prolong the cultivation cycle unnecessarily.

12.2.6 White Plaster Mold

White plaster is caused by *Scopulariopsis fimicola* and can reduce or delay harvest, as it competes with the mushroom mycelium. Due to its nutritional requirements, *S. fimicola* grows very well on alkaline compost, with a pH higher than 8. These conditions can occur when the compost has been too wet during phase I of composting, as well as when a small amount of gypsum has been added (calcium sulfate). The emergence of mold is also linked to low temperatures and a short duration of pasteurization.

Its presence is most common early in the growth cycle due to the use of materials from the previous year's composting. When it does occur, it is often found on most packages of substrate made from the same pile and pasteurization room. If the attack is strong, it can significantly delay incubation of the mushroom mycelium.

White plaster can appear at the end of phase II of composting as small irregular patches of white aerial filaments that grow on the surface of compost, which acquires a black color and a sticky texture (Figure 12.13). The aerial hyphae combine a few days later, taking on the appearance of plaster. The colonies can grow thick and quickly throughout the compost, colonizing large areas of the surface. Afterwards, it can grow from the infected area of the compost to the surface of the casing. In areas colonized by plaster, the mushroom mycelium does not develop. White plaster has also has been detected in the incubation stage and induction of fruiting on the compost and casing mixture. The critical period is from the end of phase II of composting and during incubation and induction of fructification.

12.2.6.1 Control

Strict enforcement of hygiene is required when the presence of white plaster is detected on compost or the casing material.

- During the composting process, mix the piles well and avoid anaerobic zones.
- Ensure that the whole mass of compost receives good pasteurization.
- Ensure that the levels of ammonia during phase II of composting are above 450 ppm.

Figure 12.13 White plaster mold on the compost surface. (*See color plate section for the color representation of this figure.)*

- When the mushroom mycelium begins to develop through the compost with patches of plaster, the nutrient medium becomes more acidic and gradually the mushroom mycelium overcomes the white plaster. In order to speed up this process, moistening the surface of the compost or casing material is recommended using water and vinegar, in order to acidify the medium as quickly as possible.

12.2.7 Brown Plaster Mold

This disease is caused by *Papulaspora byssina*, which shares the same nutritional requirements and compost characteristics as white plaster mold, so it also grows very well at pH values above 8. The same curative measures are also applicable. In fact, any differences are reduced to the appearance and subsequent evolution of both plasters, since white plaster does not change color during its development while brown plaster does.

Brown plaster occurs on the surface of the compost during incubation. At first it appears as white spots that change to brown from the center of the colony toward the periphery, several colonies sometimes coalescing. The spots have a granular appearance, since the fungus that causes them is characterized by the presence of bulbils. After casing, *P. byssina* grows through this layer to cover the surface of the casing material (Figure 12.14). In areas colonized by brown plaster, its presence delays incubation of the mushroom mycelium.

P. byssina is easily recognizable since it is a mass of brown spherical structures (bulbils) on the compost and casing material. It is a coprophilic fungus that prefers damp and alkaline composts, with a high C/N ratio. The critical period is from the end of phase II of composting and during incubation and the induction of fructification.

The presence of brown plaster in the compost is detected by visual inspection of the crop surface. Tracking can be carried out by counting and locating the spots of brown plaster detected in a mushroom farm.

12.2.7.1 Control

Following strict hygiene and environmental control measures is the best way to fight the disease.

Figure 12.14 Brown plaster mold on the casing surface. (*See color plate section for the color representation of this figure.)*

- Mix the piles well and avoid anaerobic zones during the composting process.
- Ensure that the whole mass of compost receives good pasteurization.
- Ensure that the levels of ammonia during phase II of composting are above 450 ppm.
- When the mushroom mycelium begins to develop through the compost with patches of plaster, the nutrient medium becomes more acidic and gradually the mushroom mycelium overcomes the brown plaster. In order to speed up this process, moistening the surface of the compost or casing material is recommended using water and vinegar, in order to acidify the medium as quickly as possible.

12.3 Bacterial Diseases

12.3.1 Bacterial Blotch or Brown Blotch

Traditionally, the bacterium *Pseudomonas tolaasii* has been considered the main causal agent of this disease, although it has also been linked with *P. reactans.* Bacterial blotch is characterized by slightly concave superficial shining brown-stained lesions of rounded or irregular shape on the mushroom caps. In favorable environmental conditions, the lesions, initially small and separated, coalesce, affecting large areas of the pileus (Figures 12.15, 12.16). The browning affects only the external layers of the cap tissues, and is restricted to 2–3 mm below the surface of the cap. When the surface of the carpophores remains wet the bacteria multiply rapidly and affected mushrooms become sticky to the touch. Both cap and stalk may be affected. The symptoms can be detected from the beginning of the harvest period until the end of the cycle. Sometimes, the first carpophores that fructify already display brown stains in the early stages of development and infect those that emerge a little later due to the proximity of the caps. Sometimes the carpophores seem healthy and, after harvesting, they develop the disease during storage. This may even occur when they are stored in refrigerated areas, because the bacteria can also grow at low temperatures. Once harvested, these mushrooms deteriorate more quickly when stored under high relative humidity conditions and packaged in trays covered with plastic films.

Pseudomonas may be present in the composts and materials used in the casing layer. The appearance of bacterial blotch is clearly influenced by the environmental conditions of farms

Figure 12.15 Bacterial blotch causing cap spotting.

Figure 12.16 Close up view of bacterial blotch causing cap spotting.

that, in many cases, are directly related to external weather conditions. If, the relative humidity present in the farms is very high (above 90%) and temperatures fall during harvesting of the different flushes, the environmental humidity may condense on the mushrooms. In such conditions, blotch may appear in the following 3–6 hours. Therefore, relative humidity and temperature are critical factors that must be controlled to minimize the risk of an epidemic. Likewise, the disease is also favored by low temperatures on the inside of the compost, which slows down the speed of evaporation after watering. Once the disease is established in a culture, it may be spread by the hands of pickers, tools, watering, pests, and so on. Bacterial blotch is more frequently observed during spring and autumn, seasons in which temperature amplitude is greater. Environmental conditions play an essential role in the appearance and severity of the disease. Sometimes, the first indication that the crop is infected is a delay in the appearance of fruiting bodies and a reduced harvest. Yield can also be affected, and the density of fruiting bodies per area of substrate may decrease because *P. tolaasii* acts not only on the reproductive hyphae of the mushrooms, but also on the hyphae of the mycelium in the compost.

This disease can be detected by visual inspection of the surface of the fruiting bodies. However, it is necessary to identify it properly since it can be confused with the blotching caused by *Lecanicillium fungicola*, *Trichoderma aggressivum*, or *Cladobotryum mycophilum*.

12.3.1.1 Control

Compliance with strict hygiene and environmental control measures are the best ways of fighting the disease.

- Remove all traces of carpophores off the culture surface.
- Improve evaporation from the culture surface and from the surface of the carpophores.
- Avoid low temperatures in the compost.
- Remove the water that remains on the surface of the fruiting bodies.
- Include a drying cycle (2 hours) after irrigation.
- Precisely control the temperature so that the dew point is not reached. Avoid temperature fluctuations.
- Prevent flooding of the casing material during the crop cycle.
- Store all tools in a pollution-free area.
- 0.3% calcium chloride can be added to irrigation water.

12.3.2 Internal Stipe Necrosis

The causal agent of internal stipe necrosis (ISN) is the bacterium *Ewingella americana*. The symptoms of ISN appear as a variable browning reaction in the center of the mushroom stipe. Examined in longitudinal section, the brown tissue extends from the base of the stalk to the cap, but rarely penetrates the cap tissue (Figure 12.17). Affected mushrooms may be wet in appearance, but frequently, at harvest, the brown tissue is dry and has completely collapsed, leaving a hollow center (Figure 12.18). In all cases, symptoms are only visible upon harvesting.

The occurrence of ISN has occasionally been associated with water-logging of the mushroom stalks at an early stage in their development, for which reason it is important that good evaporation from the bed surface be maintained at all times. An abnormally low compost temperature after the primordia of the first flush have appeared may also encourage the proliferation of *E. americana*. Although the disease may occur in many mushroom crops, its incidence does not usually exceed 1%.

12.3.2.1 Control

The critical period, after casing, is during incubation and the induction of fruiting. It is therefore recommended that evaporation from the casing be maintained and to avoid water-logging of the casing material. It is also possible to apply chlorine (155 ppm) to control the bacterial population.

Figure 12.17 Internal stipe necrosis caused by *Ewingella americana*.

Figure 12.18 Internal stipe necrosis. Mushroom with a hollow center.

12.4 Viral Diseases

12.4.1 La France Disease or Die-Back

In the epidemiology of La France disease, the mushroom spores and fragments of mycelium that are spread through the air play an important role. In fact, there is a close relationship between the appearance of virus-diseased mushrooms and the opening of the mushroom cap. The means of virus transfer are: mycelium to mycelium, through the merging of hyphae, and through the spores of mushrooms that germinate and transfer the virus to healthy mycelium again through anastomosis. This is the main reason for which extra precautions must be taken when carrying out operations with phase III compost, or when using techniques such as CACing (the addition of compost already colonized by the mushroom mycelium in the casing material to favor a quick incubation). All stages of mushroom growing can be considered critical, from the manufacture of compost to the end of the harvest.

Several symptoms associated with mushroom virosis have been described, although they are hardly ever all seen at the same time. In its most severe form, the disease causes a delay in the fruiting of mushrooms. The main symptoms are usually deformations in the fruiting bodies, such as elongation of the stipe, tilting of the cap, thickening at the base of the stipe, and tiny caps on slim or normal size stipes. This syndrome is known as "drumstick," and can be confused with mushrooms grown in an atmosphere with a high level of CO_2 (Figure 12.19). These mushrooms are usually weakly anchored into the casing, and their veils open prematurely, favoring an early discharge of spores. In addition, mushrooms show a few long brown spots in the internal part of the tissue. Sometimes the gills of affected mushrooms are poorly developed and take on a bright color. The symptoms caused by the virus can be confused sometimes with those produced by mummy disease.

The affected crops adopt an irregular appearance with poor mycelial growth on the casing. Eventually, the mycelium ends up being weaker and degenerates, leaving the compost damp. In many cases, the casing shows areas completely devoid of mushroom mycelium, a situation that some competing fungi might take advantage of to colonize these areas.

Figure 12.19 Mushrooms affected by La France disease (drumstick symptom).

The mushrooms may mature early, with premature opening of the cap. Some affected crops do not show clear symptoms, but suffer losses during harvest. When crops are affected in an early stage, crop losses are considerable. A later infection reduces crop losses, although the affected crops can still be a source of disease for other crops.

12.4.1.1 Control

It is necessary to minimize or eliminate the possibility of the virus from entering new crop cycles, in any of their stages, either by spores or fragments of contaminated mycelium. The hygiene program should be based on cleaning and disinfecting surfaces, machinery, and workers' clothing.

- Increase temperature properly during phase II, carry out pasteurization.
- Filter air during phases II and III of compost preparation.
- Use overpressure in the spawning area. Restrict the movement of workers in the spawning area.
- All machinery used in the composting factory must be washed and disinfected prior to use.
- Casing materials should be stored in a clean area.
- When possible, perform an effective cooking-out (steam treatment) of the crops.
- Treat with steam the chambers used for the production of phase III compost.
- Use filters in the mushroom farm.
- Use sheets of paper or plastic on the surface of the substrate during the incubation stage, to prevent spores falling on the compost.
- Take maximum care with ruffling of the layer of casing and "CACing," as they can favor the spreading of fragments of mycelium.
- Returnable plastic containers should be cleaned before being used.
- Collect the immature carpophores before the releasing of spores.
- Alternation of varieties to reduce the level of virus inoculum.

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13

Harvesting and Processing of Mushrooms

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13.1 Introduction

Harvesting is one of the main operations at commercial mushroom level. Harvesting has a significant impact on:

- Quality of mushrooms
- Yield obtained from the crop
- Production costs

Generally, harvesting takes place after the pinning stage, when carbon dioxide content of the growing room is lowered by introducing fresh air. At this stage mushrooms develop from the pins (5–8 mm) that have formed during the spawn run in the casing layer from the rhizophorms. These pins continue to grow and are harvested during the growing stage depending on the type mushrooms required (baby buttons, closed caps, open caps). In general, mushrooms can be picked 18–21 days after the casing layer has been spread on top of the compost (Wuest et al., 1982). After this period, mushrooms are harvested intensively for 3–5 days, this period varies according to cultivation practices (temperature, relative humidity) and cultivar of mushroom used. These periods are called flushes, crops, or breaks. Once all the mushrooms from a flush are harvested, the mycelium remains unproductive for 5–7 days when a new flush is generally ready for harvesting (second flush), this process can be repeated several times and 5–6 flushes can be obtained from the same compost batch (Beyer, 2003). However, on each flush, the yield drops significantly. Approximately, for each kg of mushrooms harvested in the first flush, only 0.6 kg are harvested on the second flush and 0.25 kg on the third flush (Beyer, 2003).

The number of flushes and yields harvested in mushroom farms is highly dependent on numerous factors, the business model being the most important one (Van Griensven, 1988). A higher number of flushes per crop increases the probability of crop diseases and pests affecting the crop in terms of quality and yield. During the harvesting process, different specifications of mushrooms are picked depending on their degree of maturation. Different product specifications appeal to different consumers, for example, Continental European consumers prefer closed mushroom caps while in the UK, Ireland, and North America consumers also appreciate large (80 mm) fully open caps called breakfast flats (for white) or Portobellos (for browns). Therefore, harvesting practices need to be changed depending on the type of product on demand. This is a clear example for large mushrooms (flats, Portobellos), which only occur

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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during the first flush. This has implications on the managing and harvesting practices of the crop/mushroom farm. A well-managed harvesting system is able to maximize the yield of the crop independently of the type of product required by consumers. Over the years, mushroom farms have introduced improved harvesting techniques and management systems to achieve high yields independently of specification on demand. Mushroom harvesting can be carried out manually preferably for mushrooms to be sold in the fresh market, or they can be mechanically harvested, which is mostly carried out for processed foods (i.e., canning).

13.2 Manual Harvesting

Mushrooms intended for the fresh market are solely harvested by hand. Manual harvesting ensures excellent quality mushrooms with an adequate shelf-life and appearance (Van Griensven, 1988), regardless of the importance of compost and casing qualities, which need to be consistent to allow a standard growing procedure. Growing operations prior to first flush (i.e., filling, casing, ruffling watering, and CO_2 levels, etc.) lead to optimal number of pins on the beds, which subsequently improves the manual harvesting process. Access to good quality mushrooms is hampered by over-pinned beds, inconsistencies within houses, and deep pinned top shelves. Nowadays, many mushroom farms focus on improving the harvesting and handling skills of pickers through extensive in-house training programs. The quality of mushrooms is readily affected from the way mushrooms are harvested. Poorly harvested mushrooms will bruise and discolor quickly. Therefore, manual harvesting has a direct impact on quality during post-harvest storage and shelf-life of mushrooms (Van Griensven, 1988). One of the most important skills required for a trained picker is their ability to graze a harvest correctly. This involves identifying the correct mushroom to be harvested at each pass and to pick them in a single handling movement to avoid bruising. The fruit body is hold firmly with the fingers and twisted gently at the base of stipe while simultaneously pulling upwards. The stipe is regularly trimmed with a small and sharp knife to remove the basal attachment of casing soil and mycelia (Van Griensven, 1988).

Mushrooms are carefully placed into plastic containers and weighed. Once picked into the containers fresh mushrooms are chilled to temperatures of 4–5°C and in relative humidity of above 90%. This chilling process is carried out for two reasons: (1) to reduce respiration rates by lowering temperatures and (2) to reduce transpiration rates by increasing the relative humidity of the storing environment. Without these conditions, mushrooms would inevitably deteriorate within a short period of time (1–3 days). This deterioration is clearly evidenced by a loss of weight, which creases and shrinks the smooth surface of the mushroom changing its color from white to a browner/off-white color (Braaksma et al., 1998). Other quality deterioration includes opening of the cap, loss of moisture, and loss of texture, aroma, and flavor. Other less marked changes include the expansion of the pileus by growth of gills and elongation of the stipe during post-harvest storage (Braaksma et al., 1998).

The shelf-life of mushrooms can be extended primarily by: (1) lean post-harvesting fast chilling and maintaining controlled chain supply storage conditions and (2) use of appropriate packaging formats to minimize the impact of changes of environmental conditions on quality attributes of mushrooms.

The impact of post-harvest storing conditions on quality of *Agaricus bisporus* was evaluated by storing mushroom for 6–8 days, at various controlled temperature conditions (3.5–15°C) and measuring the instrumental textural hardness and color of the mushroom cap for different product batches (Mohapatra et al., 2010). Mohapatra and coworkers showed that storage temperature and time had a significant impact on color and texture deterioration. By lowering

storage temperature, the extent of the browning after storage was reduced and in addition the rate of textural hardness was slowed down.

The browning developed during bruising and storage of mushrooms is due to a series of biochemical reactions dominated by action of polyphenol oxidase enzymes (PPO). Among PPO enzymes, particularly the catechol oxidase and laccase oxidase diphenols into corresponding quinones (Aguirre et al., 2009). Quinones polymerize into dark colored melanins. Browning mainly occurs in the surface of the mushrooms, where levels of phenols and polyphenol oxidase are higher than in other parts of the fungi (Aguirre et al., 2009). It has been suggested that this browning is also enhanced by the action of bacteria naturally present in the surface of mushrooms as washed mushrooms tend to be less affected by browning (McConnell, 1991; Sapers et al., 2006; Simón et al., 2010).

Burton and Noble (1993) studied the influence of flush number and the effects of controlled bruising treatments on mushroom quality during storage at cool (5°C) or ambient (18°C) temperatures. Results showed that flush 1 mushrooms tend to discolor more during post-harvest storage at 18°C from other flushes. This was assumed to be due to a higher activity of tyrosinase. In addition, flush 2 mushrooms are less susceptible to discoloration but once bruised the rate of discoloration was not different from other flushes (even if this discoloration might be due to different reasons – physiological versus pathological). Burton and Noble also observed that weight loss during post-harvest storage was not significantly different between flush 1 and 2 but that flush 3 had a higher rate of weight loss. Evaporative weight loss in different flushes was assumed to be due to differences in surface area of hyphae (and consequently of hyphal density on fruiting body) between flushes.

When mushrooms are kept at moderate mild ambient temperature (15–20°C) they can lose up to 5% of their weight in 24 hours This circumstance reveals how perishable mushrooms are and how challenging it becomes to maintain their quality during shelf-life after post-harvest and the commercial implications that a lean process might have on the industry.

Mahajan and coworkers (Mahajan et al., 2008a) further investigated the effect of the environmental conditions that have an impact mushroom quality during post-harvest storage. Mahajan's work showed that relative humidity, temperature, and gas composition are the most important factors to reduce transpiration rate and subsequent deterioration. Relative humidity (RH) is considered to be the most significant factor (Mahajan et al., 2008a). Low RH levels promote a higher transpiration rate (200–500 mg kg^{-1} h^{-1} at 20 ± 1°C) and higher weight loss (Mahajan et al., 2008a). On the other hand, high RH levels can promote microbial spoilage and color deterioration. Therefore, the control of RH and temperature are essential to avoid this deterioration. In addition to RH and temperature, gas composition plays an important role. It is well documented that the use of modified atmosphere packaging can reduce the respiration rate of vegetables and thus increase their shelf-life. This has been applied successfully to some vegetables such as pre-packed salad leaves, soft fruits and berries, and potatoes. However, the high respiration rate of mushrooms makes post-harvest storage a true technological challenge (Cliffe-Byrnes et al., 2007). Micro-perforated packaging films are commonly used in fresh produce packaging to obtain an appropriate balance between O_2 and CO_2 gas permeability and achieve equilibrium atmosphere. Modified atmosphere packing (MAP) for fruit and vegetables generally consists in maintaining levels of O_2 and CO_2 relatively low (between 5 and 10% O_2 and below 12% of CO_2). However, low levels of oxygen and temperature fluctuations on the cold chain can eventually lead to the development of *Clostridium botulinum* (Notermans et al., 1989). The presence of *Clostridium* is a considerable heath risk. Therefore, the application of MAP in mushrooms has been limited (Caleb et al., 2013). Most commercial applications are based on passive MAP rather than active MAP. Although the use of MAP for mushroom packing has shown promising results, with reported

shelf-life extension for 8–14 days (5% O_2 and 10% CO_2) depending on storage temperature, the microbial risk still persists (Ares et al., 2007; Singh et al., 2010). It is fair to say that mushroom producers have not widely adopted these technologies for the packaging of mushrooms (Briones et al., 1993).

In most mushroom storage facilities, temperature is controlled but RH is not. There are practical difficulties in maintaining high RH in large storage rooms within a narrow range. At high RH, a small fluctuation in temperature (±0.5°C) can result in condensation on cool surfaces. However, increasing the RH of the storage environment is not sufficient to prevent moisture loss. The temperature of the mushrooms must be close to the storing temperature (Mahajan et al., 2008a).

Once mushrooms are packed in plastic containers and wrapped or packed in plastic bags, the RH of the headspace inside the pack increases during post-harvest storage (Ares et al., 2007; Singh et al., 2010). This occurs because the respiration rate for mushrooms is higher than the water vapor transmission rate of the materials used for packaging mushrooms (mostly polymeric, such as polyethylene, polystyrene, polypropylene, or polyvinyl chloride). The water molecules generated as a product of physiological respiration of mushrooms do not escape through the packing film and remain inside the package. This process enhances the water vapor pressure to near-saturation conditions (RH ~100%). In these conditions, condensation may occur inside the package with small temperature fluctuations. When condensation occurs on the surface of mushrooms, microbial growth is enhanced that leads to sliminess, deterioration, and browning (Mohapatra et al., 2010).

Recent studies have focused on the management and control of RH packaging solutions. The use of packaging materials that can absorb moisture represent an opportunity to improve the shelf-life of mushrooms, by delaying deterioration due to condensation inside the pack. Moisture absorbers used for other delicate fresh produce such as berries have been used to remove excess moisture in headspace (Mahajan et al., 2008b).

Commercial moisture absorbers either have low absorption capacity and/or absorb moisture at a fast rate at the early stage of shelf-life period, making them unsuitable for packaging mushrooms. Mushrooms need a high moisture holding capacity absorber with a slower rate of moisture absorption. Mahajan (Mahajan et al., 2008b) formulated fast absorbing moisture materials such as $CaCl_2$, KCl and sorbitol, and slow absorbing bentonite in different proportions to determine the best combination for packing mushrooms. The results showed that the best mixture was found to be a 55% (bentonite) and 45% fast absorbers (0.25 and 0.2 g sorbitol and $CaCl_2$, respectively). This mixture remained active after 5 days and overall the appearance of mushrooms improved with the use of around 2% (w/w) absorber (Mahajan et al., 2008b).

More recently, the use of relative humidity-regulating package systems by directly incorporating a water absorbing substance (NaCl) in the packaging material for mushrooms has been explored (Rux et al., 2015). The packing studied was a multi-layered material, and the absorbing layer was developed as a mixture polypropylene with different percentages of NaCl (6, 12, and 18%). Humidity-regulating tray maintained a stable RH (93%) inside the package and it absorbed 4.1 g of water vapor within 6 days at 7°C and 85% RH storage conditions. Humidity-regulating tray maintained the quality of mushrooms better compared to control-PP tray, but it absorbed only 4.1 g of water vapor in 6 days, which according to researchers was not enough to completely prevent water condensation in the package headspace for mushrooms. Mushrooms can also be processed, which will significantly increase the shelf-life of the food product, but at the expense of other properties (nutritional, organoleptic). Mushrooms intended for processing are frequently harvested mechanically as they don't require being selected and picked as the ones for the fresh market.

13.3 Mechanical Harvesting

Since the late 1970s, mechanical harvesters have been introduced and widely used in the mushroom industry. The mechanization process has been driven and implemented by countries in which the cost of labor represented a significant percentage of the costs of production (usually >40%). Mechanical harvesting of mushrooms allows the producer to harvest in minutes the same crop area that would take days to manually harvest completely (Azoyam, 2004).

Mechanical harvesting can decrease significantly the harvesting time, for example, some harvesters can pick 1000 kg of mushrooms an hour versus 30 kg per man-hour in manual harvesting. However, mechanically harvested mushrooms are of lower quality than manually harvested mushrooms. Mechanical harvesting is not selective and it is very harsh on the quality of mushrooms (Van Griensven, 1988). Mushrooms mechanically harvested are bruised and if not processed immediately they brown within one hour of harvesting. Consequently, mechanically harvested mushrooms are regularly used for canning and/or processing purposes. Mechanical harvesters are usually installed in similar types of equipment as mechanical casing rufflers used in Dutch shelving growing systems. The harvester is mounted in the shelf and it slides from one end to the other slicing all mushrooms in the bed. Mushrooms are transferred into a conveyer belt, washed, and processed immediately.

The crop managing practices for mechanical harvesting are also different to the manually harvested ones; after mechanically harvesting mushrooms, the stalks are removed from the growing beds. This is performed either by allowing the stalks to grow slightly, introducing fresh air to dry them and using the mechanical harvester again or by using a specific machine to rake the stalks off the bed. The dry conditions are useful to avoid the incidence of green mold (*Trichoderma* spp.) on the growing bed, but more often beds are treated in between the flushes to avoid any pests. After this stage the pins for the next flush tend to appear. Mechanical harvesting is carried out at a different time to manually harvested mushrooms. Yield and quality of mushrooms is inversely correlated, therefore the later the mushrooms are harvested, the bigger the yield but at the same time the poorer the quality of the mushrooms harvested (Svekker, 1971; Vedder, 1979).

Because of the characteristics of mechanical harvesting, different mushrooms strains are regularly grown specifically for automatic harvesting (Van Griensven, 1988). Off-white strains show a better performance in mechanical harvesting. Nevertheless, technological advances in computer image analysis and robotics have introduced a further advanced harvesting system that remains at the interface between manual and mechanical handling.

13.4 Automatic Harvesting Systems

Harvesting is the only step in commercial mushroom cultivation that is not yet mechanized. The technical feasibility of automatic harvesting systems has been assessed since the 1990s. The results revealed that in many cases it was economically feasible to use robots in the mushroom harvesting process: mushroom harvesting can represent between 15 and 30% of the production costs of mushrooms. Therefore, since then, attempts have been made to address the challenge of achieving a fully automatized harvesting system that can carry out the tasks of locating, identifying and selecting, picking, trimming, and transferring the mushrooms into a packing line. Although there are some commercially available robots that can achieve harvesting in different degrees, it is fair to say that automatic harvesting systems have not been rolled out in the mushroom industry and most innovations have been incremental rather than radical or disruptive.

Reed (Reed and Crook, 1995), developed a computer based vision system and combined it with a series of algorithms in order to successfully locate and size mushrooms in a growing bed. The system also had a prioritization algorithm that determined the picking order in a mushroom growing bed so as to maximize picking success rate. The picking device reproduced the dexterity of human harvesters by using a suction cup device employing a bend and twist action. The main challenges for this research were the selection process through computer image analysis and the picking of mushrooms in highly clustered crops, which precluded the picking device to successfully pick given mushrooms without damaging neighboring ones.

The original design by Reed was further developed to a more robust harvesting rig (Noble et al., 1997). This new design incorporated two new advantages: (1) mushrooms could be picked by a combination of bend first, then twist to reduce contact with neighboring mushrooms and (2) a strategy to prioritize the picking of mushrooms to improve pick success rate. This harvesting system has been improved over several iterations (Reed et al., 2001).

Although a few methods exist for use of digital cameras for locating and measuring cap diameters of mushrooms in a mushroom bed before harvesting the mushrooms with a mechanical picker, most of these methods cannot select and subsequently pick mushrooms depending on separation and stagger processes as done in manual harvesting. In recent years, several patents have been filed describing harvesting aid devices of this type (NL 86/00887, US 5,058,368, US 5,471,827 US 8,033,087, and US 2005 0268587).

Although some developments have been achieved on automated methods and systems for harvesting mushrooms from a mushroom bed, a method where both separation and stagger harvest takes place automatically and in a controlled way still remains to be invented. The separation process is the harvesting process by which two types of mushrooms are harvested: the large size mushrooms and the smaller size mushrooms. The aim of picking the smaller mushroom is to provide room for adjacent, larger mushrooms to grow to a larger size. The staggering process is the harvesting process that considers the generational differences of the mushrooms in the growing bed and ensures that there are mushrooms available to harvest consistently through the cropping process. A correct and strategic balance between the separation and stagger processes is important in order to maximize the yield of the crop efficiently. Variations in the harvest result in terms of yield and quality of crop picked occur between operators. A successful automatic harvesting system depends on the ability of the system to carry out both processes efficiently.

The patent WO 2015/164958 describes an invention that provides more refined harvesting based on separation and staggering processes. The method digitally measures the cap diameters of mushrooms growing in a mushroom bed, whereby any mushrooms with a cap diameter equal to or greater than a pre-set value are to be picked. The system is able to locate the centroid positions of each mushroom in the bed. Then by calculating and comparing the centroid-to-centroid distances for two mushrooms the system can determine if there are interfering mushrooms between them in a clump that would require thinning. This recognition system can be used to support pickers with which mushrooms to pick, reducing the picker to picker harvesting variation. However, the system can also be complemented with a robotic harvester that is configured with the image system and determines which mushroom to pick from the identified clumps of mushrooms.

The US Patent US2013/0340329 describes a harvesting device that facilitates the harvesting of mushrooms growing on a substrate or soil. The harvesting device comprises: (1) a harvesting conveyer capable of manually picking mushrooms in a container and (2) a bridge that transports the harvesting conveyer over the whole length of the growing bed. The harvesting device can simultaneously determine one or more characteristics of the individual mushrooms

growing in the bed, such as size, weight, or color. Mushrooms are manually picked (with stalks) into the principal conveyer belt and their stalk is subsequently removed. The invention uses a computer vision system that records the images of the picked mushrooms, and is able to calculate/predict by means of a suitable algorithm different characteristics of the mushrooms (such as weight based on the size of the cap). The algorithm can also sort the picked mushrooms on the basis of size classes. Once the mushrooms are placed in the conveyer, a "pick and place" robot transfers selected mushrooms into a punnet and when filled, the punnet is discharged into a side conveyer belt for packaging. The manufacturers claim that the cost of manually harvesting the mushrooms is substantially reduced with a 300% potential increase on harvesting performance (https://www.youtube.com/watch?v=bscKjv0LCJ0, accessed April 2016).

13.5 Washing Mushrooms

Over the last 30 years, the consumption of ready to eat fresh produce has become more popular. Ready to eat fresh produce offers convenience and mostly if it is pre-cut and/or washed products. Ready to eat sliced and quartered mushrooms are present in many countries and the microbiological safety criteria has become an important aspect for these foods. Mushrooms are washed to eliminate casing residues from the product, extend shelf-life, and to minimize the risk of presence of human pathogens related to fresh produce (mostly *Escherichia coli 0157:H7*, *Salmonella* spp., and *Listeria monocytogenes*). In most cases the mere presence of one of these pathogens is enough to trigger a product recall. Most fresh mushroom recalls have been related to *Listeria monocytogenes.*

Listeria monocytogenes is of significant concern to the agricultural and food processing industry because of its ability to grow and persist in the cool and moist environments characteristic of the fresh produce supply chain (Viswanath et al., 2013). Listeriosis is a disease with a very high mortality rate. Although there have been no listeriosis outbreaks attributed to fresh mushrooms in the United States and Europe, microbiological surveys (Food Safety Authority of Ireland, 2006) and recalls of *L. monocytogenes* presence in mushrooms (*Agaricus bisporus*) have occurred in the past. Also, a Listeria challenge study has been carried out on *Agaricus bisporus* mushroom following EU guidelines for these type of analyses (EURL, 2014). Results from this challenge study have shown that button mushrooms do not promote the growth of *Listeria* on their surface above 100 cfu/g at specific storage conditions as required by the European Commission (EC No 2073/2005).

Aqueous solutions of sodium metabisulfite are the most frequent method used to wash mushrooms. Sulfite wash is well known for improving the whiteness and overall quality of the mushrooms. Sulfite delays but does not inhibit browning due to microbial spoilage. Sulfites have been banned since the 1980s in some jurisdictions and are often very unpopular because of their association with potential allergic reactions and other symptoms (McConnell, 1991). Since then, several alternatives have been explored to wash mushrooms.

A comprehensive review of potential wash solutions for mushrooms including hydrogen peroxide, potassium sorbate, sodium salts of benzoate, EDTA (ethylenediaminetetraacetic acid), and phosphoric acids was carried out by McConnell (1991). The results showed that shelf-life extension occurred when antimicrobial and antioxidant compounds were combined. McConnell developed an aqueous preservative wash solution containing 10,000 ppm H_2O_2 and 1000 ppm EDTA. The H_2O_2 is a bactericidal agent that causes DNA oxidative damage, while EDTA is a strong chelating agent, which binds strongly to copper, a functional cofactor of the mushroom browning enzyme, tyrosinase. By chelating copper the tyrosinase activity and the discoloration of mushrooms are reduced.

Later Sapers (Sapers et al., 2006) included H_2O_2 into a two-stage mushroom wash, employing 10,000 ppm H_2O_2 in the first stage and 2.25 or 4.5% sodium erythorbate, 0.2% cysteine-HCL, and 500 ppm or 1000 ppm EDTA in aqueous solution in the second stage. H_2O_2 treatments are equivalent to or better than metabisulfite treatments in terms of whiteness and shelf-life (Sapers et al., 2006).

A two-step washing method for mushrooms based on a first step high pH antibacterial wash followed by a neutralizing wash has been patented (US Patent 5919507). The neutralizing wash contained a buffered solution of erythorbic acid and sodium erythorbate. Other browning inhibitors, such as ascorbates, EDTA, or calcium chloride, were identified as suitable ingredients for addition to the neutralizing solution. The process also helped remove debris and delayed microbial spoilage of fresh mushrooms.

A method for washing and preserving mushrooms characterized by combining: (1) precooling, (2) washing with a mixture of preservatives, and (3) drying steps while maintaining temperature controlled in the range of 3–7°C has been also patented (EP2476316 A1).

13.6 Canning Mushrooms

Canning is a straightforward solution to the rather short and problematic shelf-life of fresh mushrooms. Canned mushrooms used to (and still do in many countries) surpass their fresh counterparts in volume and consumer preference. However, new consumer trends have seen a decline of canned mushrooms in favor of fresh. Increasingly, consumers prefer fresh or minimally processed vegetables for idealistic and nutritional reasons. Canning remains a large section of the market and its consumption is still popular. Canned mushrooms lose approximately 25–30% of their fresh weight when subject to the blanching process prior to canning. The blanching process is standard in canning operations and aims to inactivate certain enzymes. The sterilization process after canning, which comprises of temperatures of up to 120°C at the center of the sealed can and holding that temperature to destroy thermophilic organisms, results in additional 10–15% weight loss. This is the reason why canned mushrooms tend to be smaller in size and firmer in texture than their fresh counterparts.

Some efforts have been directed toward methods of preserving mushrooms while minimizing the amount of weight loss produced by shrinkage. The US 20140057029 patent aims to minimize the shrinkage and reduce waste by inserting clean mushrooms in a plastic laminate bag and subjecting the bag to a vacuum. Mushrooms then are subjected to a mild thermal treatment (60°C) that is equivalent to blanching at atmospheric pressure. This step aims to avoid weight loss by retaining the released water from mushrooms and then further thermally treating the mushrooms in their own blanching juice. Through this process energy is saved, waste is reduced, and the taste and nutritional value of mushrooms is improved.

13.7 Conclusions

- Fresh mushrooms are a challenging product to harvest and preserve during post-harvest conditions.
- Many studies have directed their effort to improve harvesting techniques manually and using automatic harvesters.
- Physiological characteristics of mushrooms promote their rapid microbial spoilage.
- The high microbial load present in fresh mushrooms does not represent a food safety concern.

- Modified atmosphere and moisture absorbing packaging have shown a limited impact on mushroom quality over shelf-life.
- Washing and thermal treatment (canning) are viable processes to extend the shelf-life of mushrooms.

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14

Mushroom Farm Design and Technology of Cultivation

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Today, mushroom cultivation is one of the biggest money-spinning enterprises in the world and the mushroom is an important horticultural cash crop. Mushrooms, besides being a delicacy, have tremendous attributes on the basis of food value and are now recommended as a health food rich in proteins by the Food and Agricultural Organization of the United Nations for bridging the protein malnutrition gap in developing countries. Mushroom as a food is potent and important as it is produced from recyclable agro-wastes/agro-byproducts and the requirement of land is not pre-requisite in its cultivation, as it is grown indoors in protected houses with environmental control, with intensive space utilization in vertical/horizontal axis in cropping rooms.

Mushrooms, being an indoor crop, do not require arable land, except for some non-agricultural land to build the infrastructure for preparation of substrate, raising the crop, preparation of spawn, and post-harvest handling. White button mushrooms in India are grown seasonally and in environment-controlled cropping houses, and both require building of a basic infrastructure. Seasonal growing is done for 5–6 months when outside temperatures are favorable for the crop, that is, during winter months in the north-west plains and from September to April in the hills.

Seasonal growing is termed a low input-low production system and the environmentally controlled year-round growing system the high input production system; both are relevant as far as the economics of mushroom cultivation is concerned. In India, protected cropping of mushrooms was started on a modest scale in the early 1960s at Solan and later it was taken up in other regions of the neighboring states like Jammu and Kashmir, and Uttar Pradesh. Earlier button mushrooms were grown in makeshift rooms or ready-built structures with minor modifications for ventilation. The growing was done either in wooden trays or shelves and compost prepared using a long method in a single extended outdoor phase (without steam pasteurization).

Soon, with the availability of know-how and increased interest from research and development workers, mushroom farming took the shape of a cottage industry in Jammu and Kashmir, Haryana, Himachal Pradesh, and other states. Some large mushroom farms were also built in areas around Solan, more so because of proximity to the Mushroom Research Laboratory at Solan for know-how and a cooler climate prevalent in the area for growth of white button mushrooms (*Agaricus bisporus*). But today mushroom farms are being built at all elevations/places in India with improved availability of the know-how for cultivation in environmentally controlled cropping houses, specially built for the purpose.

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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More and more modifications and innovations have been perfected to suit growing conditions in India and mushroom farm design developed using locally available materials/machinery for higher productivity at lower costs (Dhar, 2005). Following the Dutch pattern of bulk pasteurization in tunnels and growing in beds laid on shelves inside environmentally controlled cropping rooms, the mushroom industry has received great impetus in the last 15 years in India. Modern mushroom growing units/Export Oriented Units have been built by big industrial houses chiefly for export. Most of these EOUs are located on the tropical plains of India.

The importance of a proper and suitable design for a commercial mushroom farm suited to conditions in India has become a necessity, and details of the farm design perfected over a period of time with special requirements of growing in India are given in this chapter.

14.1 Selection of Site and Pre-Requisites

Before selection of a site for the building of a mushroom farm is made, it is necessary to look into the following pre-requisites:

1) Training on mushroom production technology.
2) Preparation of a project report.
3) Arranging the finance.
4) Execution.

Mushrooms, being an indoor crop, involve heavy expenditure on building of infrastructure, purchase of machinery and equipment, raw materials, labor, and energy. It is very important for an entrepreneur to undergo practically oriented training for learning and understanding various stages of mushroom cultivation. There are scores of institutions in India besides DMR (Directorate of Mushroom Research, ICAR research institute on mushrooms), where basic training on cultivation of mushrooms is imparted. These are the Agricultural Universities located the length and breadth of the country, NGOs like Krishi Vigyan Kendras (Agricultural Information Centers), Council of Scientific and Industrial Research Mushroom Laboratories, and others.

After completion of the training, a detailed project report has to be prepared after a decision has been made by the entrepreneur on size of the mushroom farm and type of mushroom to cultivate. The project report should be prepared by specialists/experts in mushroom production technology. Finally, the third difficult task will be arranging the finance for the project. The execution of the project to be done under expert guidance of the mushroom expert or a consultant is another important theme. Before selection of the site, the following points have to be taken into consideration for greater operational efficiency and cost effective production of mushrooms at the farm:

1) The site should be near to the residence of the entrepreneur, for effective involvement in supervision and decision making at the farm.
2) The site should be serviced by a drivable road, or near a road, to reduce transportation costs of raw materials to the farm/finished product to the market.
3) Availability of plenty of water at the site, as the mushroom is 90% water.
4) Easy availability of raw materials at cheaper costs in the area.
5) Easy availability of labor at cheaper costs.
6) Availability of electricity at competitive prices, as power consumption is tremendous in mushroom cultivation.
7) Green cover at the site to ensure efficient working of air filters on air handling units (AHUs) with availability of clean air.

8) The site should be away from industrial pollutants like chemical fumes, coal exhaust, and other such pesticide/chemical pollutants that cause harm to mushroom production.
9) There should be provision for sewage disposal at the site.

14.2 Components of a Mushroom Farm

Farm design deals principally with the building of various infrastructural facilities required for different operations like composting, spawn making, cropping, and post-harvest handling on a piece of land in such a way that various operations are performed with greater efficiency at the minimum cost. Since mushroom growing is principally an indoor activity, hence construction of infrastructure needs to be done with skill and under expert guidance.

Button mushrooms, *Agaricus bisporus*, grow on composts prepared by aerobic fermentation of agro-wastes/byproducts like cereal straws and animal manures, unlike oyster mushrooms such as *Pleurotus* spp. and *Volvariella* that grow directly on unfermented straws/lignocellulosic materials. For production of button mushrooms, the entire process can be divided into four operations:

1) Composting (substrate preparation).
2) Spawning (sowing of seed).
3) Cropping and crop management.
4) Post-harvest handling.

For performing these operations, the following infrastructure is required to be built for establishment of a mushroom farm:

Composting unit:
Outdoor phase I composting platform/indoor bunkers or aerated chambers.
Indoor phase II in peak heating/bulk past-chamber.
Peak heating chamber.
Bulk pasteurization chamber.
Casing pasteurization chamber.
Spawn unit:
Spawn laboratory.
Cropping unit:
Seasonal cropping rooms.
Environmentally controlled cropping rooms.
Environment control, air conditioning, and forced air circulation.
Ancillary units.
Post-harvest handling unit:
Pre-cooling chamber.
Canning hall with canning line.
Packaging room.

The compost for button mushrooms is prepared using long and short methods. The long method of composting is a low technology process and does not need the building of a bulk treatment chamber for composting as the short method does. Compost by the long method is prepared on a covered composting platform on a clean cemented/brick lined floor. For short method compost making, a fully fledged covered composting yard with reinforced concrete floor and a guddy pit (leachates from compost are collected here) is required for mechanized phase I composting. For phase II of composting an insulated bulk pasteurization chamber or

peak heating chamber is required where compost is subjected to uniform high temperature treatment for pasteurization (57–59°C) followed by temperature (aerobic) controlled fermentation at 48–52°C (also called conditioning/enrichment of compost).

The bulk chamber is insulated and made leak proof from all sides, ceiling, floors, walls, and doors. The growing rooms also need a high degree of insulation to cut-off the external environment for maintaining an appropriate climate for raising a crop of mushrooms in all seasons. For seasonal growing, a normal brick walled cropping room with a brick lined cemented floor and a false ceiling will be enough to produce a healthy crop of mushrooms in appropriate season in hills or plains.

A simple forced air circulation system will be sufficient to provide necessary air changes in the room for removal of CO_2 from cropping rooms. In the case of insulated/climate controlled cropping rooms, AHUs are required to be installed for each cropping room for cooling/heating, RH, and CO_2 concentration maintenance during cropping. This necessitates the installation of cooling/heating systems for the growing rooms. A spawn laboratory and a canning unit will be required on large farms as supporting units for seed/spawn production and canning of the mushrooms produced, respectively.

14.2.1 General Layout/Location of Various Units

Some important factors to be kept in mind before selection of site for the farm are availability of clean water, regular availability of power with a three-phase facility, drainage arrangements, availability of raw materials and labor, vicinity of market, and green cover in the surrounding area. The site should be away from populated areas as foul odors emanating from the compost yard can be bothersome and a source of odor. The mushroom farm (Figure 14.1) may consist of the following units and sub-units: (1) Composting unit consisting of covered outdoor

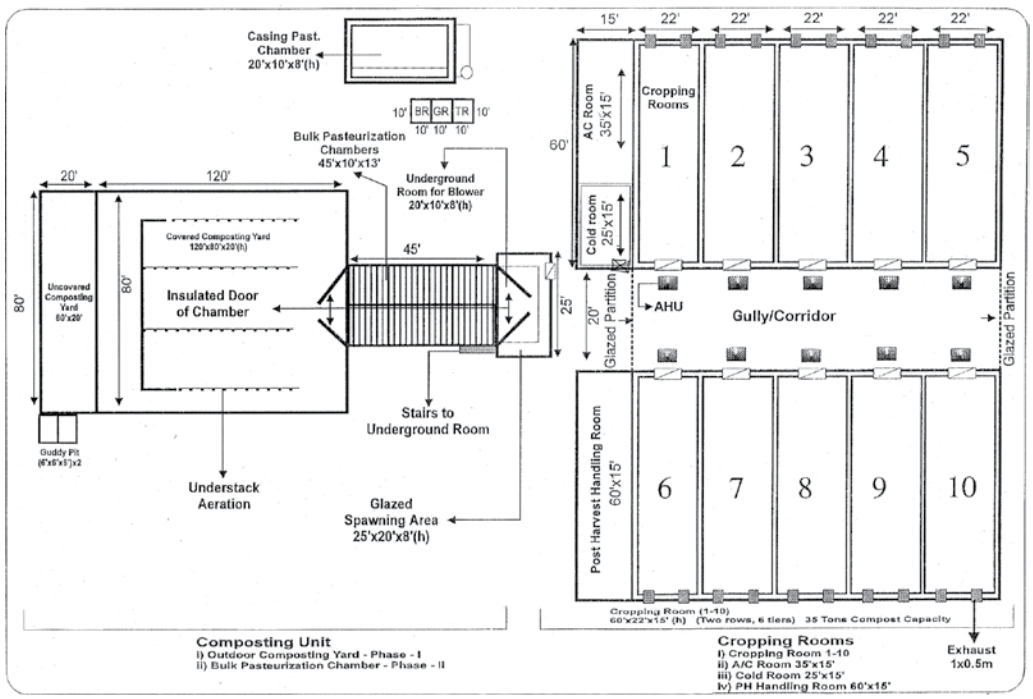
Casing Past. Chamber 20'x10'x8'(h)
Bulk Pasteurization Chambers 45'x10'x13'
Underground Room for Blower 20'x10'x8'(h)
Covered Composting Yard 120'x80'x20'(h)
Uncovered Composting Yard 80'x20'
Insulated Door of Chamber
Guddy Pit (6'x6'x8')x2
Understack Aeration
Stairs to Underground Room
Glazed Spawning Area 25'x20'x8'(h)
AC Room 35'x15'
Cold room 25'x15'
Cropping Rooms
Glazed Partition
AHU
Gully/Corridor
Post Harvest Handling Room 60'x15'
Cropping Room (1-10) 60'x22'x15' (h) (Two rows, 6 tiers) 35 Tone Compost Capacity
Composting Unit
i) Outdoor Composting Yard - Phase - I
ii) Bulk Pasteurization Chamber - Phase - II
Cropping Rooms
i) Cropping Room 1-10
ii) A/C Room 35'x15'
iii) Cold Room 25'x15'
iv) PH Handling Room 60'x15'
Exhaust 1x0.5m

Figure 14.1 General layout of 250 TPA (tons per annum) mushroom farm.

composting yard, Pasteurization chamber/peak heating room, Casing pasteurization chamber, (2) Spawn unit, (3) Cropping unit, and (4) Post-harvest unit. The composting yard is built nearer to the main road for operational convenience. The bulk chambers are built on the other side of the composting yard (away from the road) so that the distant end of the chamber opens nearer to the cropping rooms and away from the composting yard. The cropping houses are built away from the composting area for reasons of cleanliness and avoiding contamination by pests and pathogens. The casing pasteurization chamber is also built away from the composting yard or on one side of the bulk chambers. Enough space for future expansion of the composting yard, construction of more bulk chambers, and growing rooms should be left vacant for planned development of the mushroom farm in a phased manner.

The foundation of the buildings is dug on firm ground. The underground water pipes, electrical cables, and sewers are laid well before the actual construction starts. The entire site area should preferably be fenced or brick walled for security reasons. In areas where land is scarce, double floored cropping houses can be built to economize on space. The cropping rooms are generally built in double rows with a path/gully in between for various operations and services.

14.2.2 Composting Unit

14.2.2.1 Covered Outdoor Composting Platform

The composting yard (Figure 14.2) is required for phase I of composting (pre-wetting and outdoor composting). The composting yard should necessarily be a covered shed without side walls where rain will not interfere in the normal process of composting. The high roof will facilitate escape of foul gases into the atmosphere. The foundation of the composting yard should be laid on firm ground. After digging, a layer of sand 15–20 cm thick is laid followed by a layer of broken stone/brick pieces 15–20 m thick. Thereafter, a cemented floor is poured in one operation, 20 cm thick, preferably as reinforced cement concrete. The provision for understack aeration is laid in the floor in the form of perforated pipes, connected to a blower. The floor is given a run-off of 1 cm per running meter away from the bulk chamber and toward the guddy pit.

The roof of the outdoor composting platform is built on trusses or RCC pillars 6 m (20 ft) high with GI roofing. The sides are without a wall to allow the foul gases to escape into the atmosphere. The sides of the shed are closed under special circumstances when composting is done in hot summer months in tropical areas. The guddy pit is built away from the bulk chamber on one end of the platform. The guddy pit is provided with a dewatering pump and a hose.

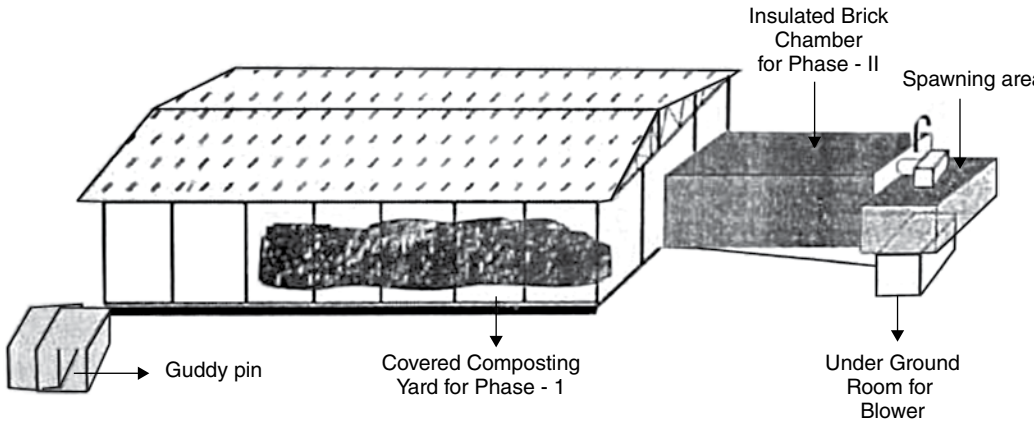

Figure 14.2 Compost yard, bulk chamber, and spawning area.

The covered composting yard should be big enough to hold compost stacks for phase I of composting, and the size of the composting yard will be determined by the number and capacity of bulk chambers. On an average one ton of compost occupies about one meter length of the compost yard, with an extra space of 2–3 m left on each side for turning with machines. Two bulk chambers will have a platform with 10–15 m width. For two bulk chambers with a 25 ton capacity each, a composting yard of 42 × 24 m should be good enough to concurrently run various operations like pre-wetting/phase I for both the chambers. It is, however, advisable to provide understack aeration for outdoor composting on the platform.

Now aerated bunkers are in use and labor and space is saved for outdoor composting in ricks. The compost yard should have a 1 m (3') wall on the periphery to prevent entry of run-off water in hilly areas in the rainy season, which can bring nematodes, insect pests, and other undesirable elements into the compost yard. The compost yard should be approachable by a drivable road on one side (away from the bulk chamber), so that the raw materials can be directly unloaded on one end of the platform, which will save the labor cost of transporting the raw materials from the road head to the compost yard. In hilly areas, a chute can be built from the road down to the compost yard to easily download the raw materials on to the platform from the drivable road, thus reducing labor costs.

Water connection with a 5–7 cm (2–3") diameter pipe should be available in the composting yard permanently with an additional portable hose pipe of 7–10 cm (3–4") diameter for use during pre-wetting. One dewatering pump with a hose should be installed in the guddy pit to pump out the run-off water for its reuse during pre-wetting. A drain should run on the two sides of the platform to facilitate periodic cleaning of the platform. Alternatively, sunken traps for drains and fresh water connection can be provided at the composting yard.

A few three-phase 15 A power connections should also be provided at the composting yard for operating machines like the automatic compost turning machine, filling line, and spawning machine. The yard should be well lit with tube lights and strong search lights to facilitate round-the-clock operation at the composting yard. An overhead water tank is necessary, particularly where water is scarce, to store water for timely operation.

14.2.2.2 Pasteurization Facility

The bulk pasteurization chamber and peak heating chamber are principally used for phase II of composting for pasteurization and high temperature controlled fermentation (also called conditioning). For this purpose, an insulated chamber is built with facility for steam inlet, blower, and controlled fresh air entry. The insulated chamber is built with the purpose of cutting off the external environment and stimulating a desired environment inside for controlled fermentation of the composting ingredients. Two types of chambers are used for this purpose: (1) peak heating chamber and (2) bulk pasteurization chamber (tunnel).

14.2.2.2.1 Peak Heating Chamber

The peak heating chamber consists of an insulated room with facility for injection of steam, air intake, and recirculation. The compost after phase I is filled in trays or racks inside the peak heating chamber and pasteurized/conditioned. This facility is more suited when smaller amounts of compost are handled. The modification of this system is what we term the "single zone system" of peak heating. In this system, all rooms on a mushroom farm are excellently insulated and provided with steam, air handling, cooling, and heating facilities. The compost after phase I is filled in racks/trays in the cropping room for all operations in a row, like pasteurization/conditioning, spawn run, case run, and cropping. All the operations are done in the same chamber for labor economy and efficient utilization of the available space. This system can be more efficient in already built structures like cold storages, and so on, where the

entire space is utilized to maximum efficiency without investing in construction of bulk chambers for composting. The single zone system is expensive and the initial capital cost is higher, as all the rooms will have to be provided with facilities for all the operations to be carried out in series. This system is in use in some of the old mushroom farms in Western Europe and most of the farms in the USA.

14.2.2.2.2 Bulk Pasteurization Chamber/Tunnel

This is a modification of the peak heating chamber with the difference that in this case compost can be handled in greater bulk quite efficiently (Derks, 1973). This is termed the "double zone system." The compost after phase I is filled into a specially built chamber, which is properly insulated and provided with steam connection and air blowing system for recirculation. The compost is filled in the chamber on top of its grated floor built over the plenum. The plenum has an air circulation duct used during pasteurization/conditioning (as shown in Figure 14.3).

The bulk chamber should be constructed at one end (away from the road) of the composting platform. One end of the bulk chamber should open into the platform and the distant end in the clean spawning area. The foundation of the bulk chamber should be dug on a firm base ground. The foundation is dug 0.5–0.6 m (1.5–2') deep, depending upon the firmness of the ground. The floor of the chamber should be laid starting with a sand layer 15–20 cm thick, followed with a layer of broken brick/stone chips 10 cm thick and a concrete floor (1:3:6); and insulation with thermocol/glasswool 5 cm thick (15 kg/m^3 density). The insulation is covered with an isolating membrane of PVC sheeting followed by 5 cm cement floor and finally the finish. Such a floor is constructed (as before) for both the cropping room and chamber. The walls should be 23 cm (9") thick (one brick lengthwise) built over the concrete foundation. The length and breadth of the bulk chamber will vary, depending upon the amount of compost to be treated in the chamber, with the height of 4 m (13'). The roof is made of 10 cm (4") thick RCC. The walls, ceiling, and the floor below the plenum are insulated with 5 cm thick insulating material (15 kg density per m^3) necessary for an effective insulating effect during pasteurization and conditioning of the compost (Vedder, 1978).

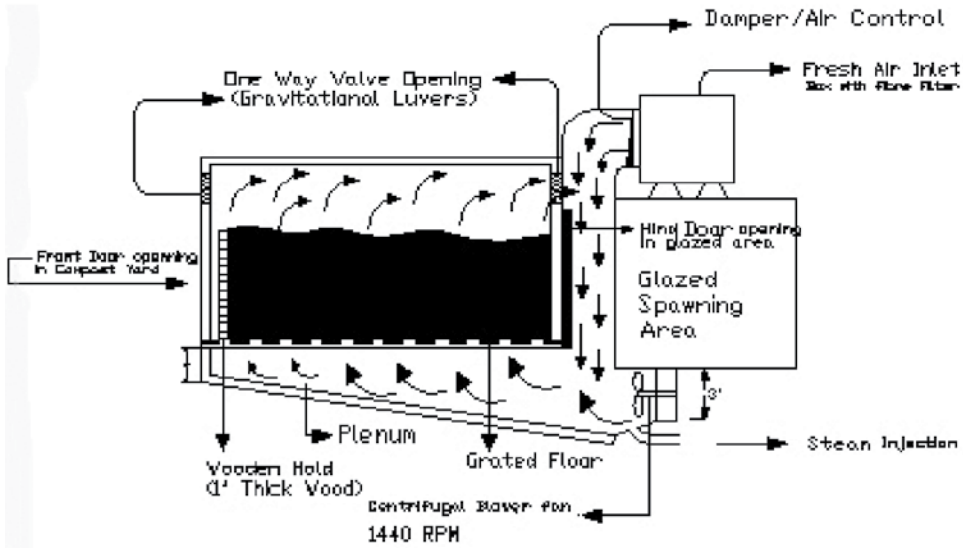

Figure 14.3 Cross section of a compost bulk pasteurization chamber (length 13 m/45', width 3 m/10', height 4 m/13').

The floor must be laid with a good run-off provided with a drain to facilitate cleaning. Air leakage in the bulk chamber must be prevented at any cost. The grated floor is laid above the plenum over the ventilation duct. The grated floor must allow the air to pass through, for which approximately 25–30% of the floor area is left in the form of gaps for ventilation/circulation of air and steam. The plenum is divided with a perforated brick wall (one or two) in the center for supporting the grated floor. The gratings can be made of wood (painted with bituminous paint), coated iron strips mounted on angle iron frame or cement if possible. If nylon nets are to be used for mechanical filling and emptying, then a cemented grated floor with appropriate RCC strength is built specially for the purpose.

The doors of the bulk chamber are made of angle iron or wooden frame with 5–7 cm (2–3") insulation in the middle and covered on both sides with aluminum sheets. The chamber will have two exhaust vents, one for recirculation exit and the other for exhaust of gases on introduction of fresh air via filtered dampers. Fungal filters of 2–3 μm are fitted on the entry points to keep out pathogenic fungal spores and other pests. The fresh air dampers are provided on top of the roof and connected with the recirculation duct for introduction of fresh air when needed. The chamber is serviced by a blower fan below the plenum, installed either in the underground room or on the side of the chamber. The blower fan size will depend upon the tonnage of compost to be handled in the bulk chamber.

A centrifugal blower fan energized by a 5–7.5 HP motor with a speed of 1440 rpm will be able to produce necessary air pressure of 100–110 mm of water level at entry point required in a bulk chamber of 20–25 tons compost capacity. The steam line is also connected at the entry point. The walls and ceiling can be damp proofed by coating bituminous paint on the inside over the cemented surface, which will also serve as an effective vapor barrier. The grated floor inside and the work floor outside should be of the same height for operational convenience. Two types of tunnels (bulk chambers) are in use, two door bulk chambers and single door bulk chambers. In the single door bulk chamber, the same door is used for filling and emptying and the other end is utilized for fixing installations (blower, etc.). In the double door bulk chamber, one door is used for filling (which opens into the composting yard) and the other for emptying (opening into the sterile spawning area).

Apart from effective insulation and damp proofing the walls/ceiling, no other specific requirement needs to be met in construction of a bulk chamber. The bulk chamber can be filled/emptied manually or by conveyer belts/machines. The use of machines for filling/emptying is labor saving, time saving, and ensures maintenance of absolute cleanliness during operations. For mechanical emptying two nylon nets are used, one fixed over the RCC grated floor and the other moving over the lower net (pulled by a winch). The compost when brought out is fed into the spawning machine where the requisite amount of spawn is mixed with the compost and the seeded compost is then poured into clean polythene bags for transport to the growing room.

14.2.2.3 Cooling in Tropical Areas of Compost in Summer Months: Special Requirements in the Compost Bulk Chamber

Cooling equipment installation is required for cooling of compost after completion of conditioning in summer months, when outside ambient temperatures are around 35–40°C. One AHU with cooling coils is installed inside the chamber or placed outside the chamber with heavy insulation. Alternatively, cooling equipment is installed with an underground blower. This additional arrangement is required only under tropical conditions when the outside ambient temperature is higher in summer months. This system is proving very effective for cooling of the compost after completion of the composting process in tropical areas.

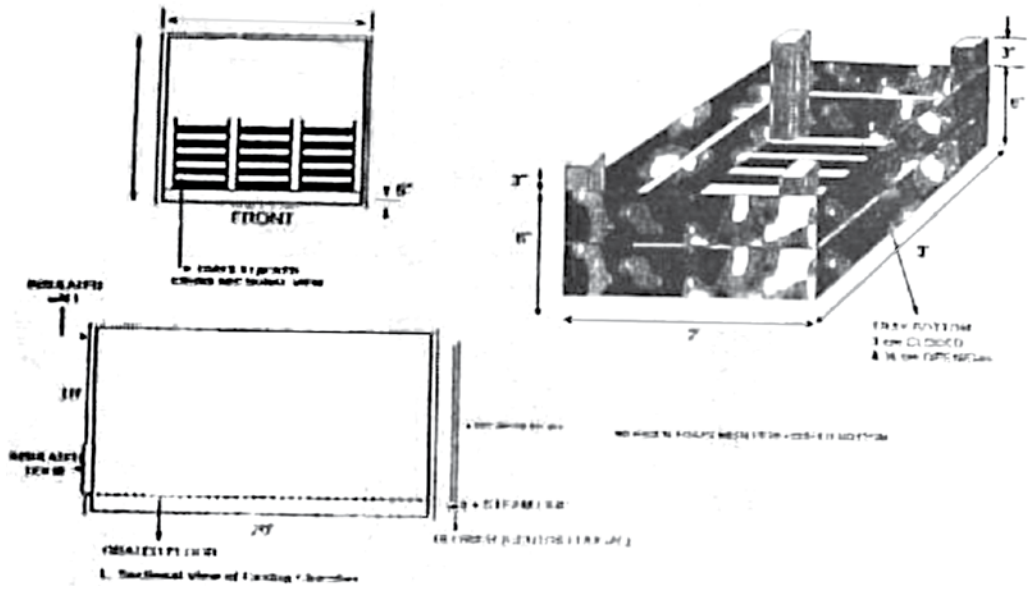

Figure 14.4 Casing pasteurization chamber.

14.2.2.4 Casing Pasteurization Chamber

The casing pasteurization chamber is an insulated chamber with a steam connection and a blower for effective circulation of steam inside the chamber for attaining correct temperatures for pasteurization of the casing materials (Figure 14.4). The size of the chamber will depend upon the size of the compost chamber and the size of the growing rooms. One chamber load should provide casing for one compost lot from each tunnel. The casing after wetting is filled into the perforated wooden/aluminum trays which are stacked one over the other inside the chamber and steam treated at 65°C for 6–8 hours. The door of the casing pasteurization chamber should also be insulated as in compost bulk chamber and made air tight by fixing a rubber gasket on the inner boundary of the door. This chamber should be built away from the composting yard to maintain absolute hygiene and cleanliness.

In India, a tremendous problem is experienced in selection and use of casing materials as peat is not available, so rotten farm yard manure (FYM) and spent compost (both 2 years old) are used as a casing medium with fairly good results. But there is a problem with salt accumulation in these materials, resulting in higher electrical conductivity. This can be rectified by washing/steeping these materials in clean water for 4–6 hours before steam pasteurization. So, construction of cemented water channels/tanks with arrangement for free flow of clean water above and drainage below is required to wash/steep the casing material before use.

14.2.3 Spawn Unit

The layout plan of a spawn laboratory is given in Figure 14.5. A total built-in area of 18 × 9 × 3.5 m (60’ × 30’ × 12’ h) should be good enough to house the entire spawn unit. This area will be divided into different work areas like cooking/autoclaving room, inoculation room, incubation room (insulated with A/C), washing area, store, office, and one cold room (heavily insulated) for storage of spawn when required. The spawn (seed) of mushrooms has to be

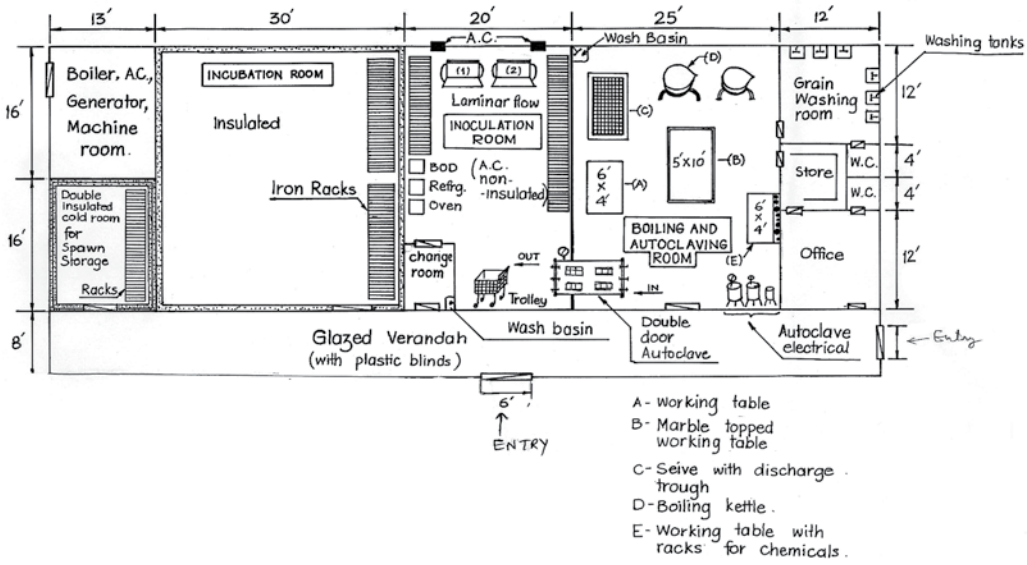

Figure 14.5 Layout plan of a spawn lab.

prepared by a specialized technique involving several steps in the series. Information about spawn production is given in Chapter 5.

14.2.4 Cropping Unit

Since mushrooms are grown indoors under a simulated environment specially created for mushroom growth, the cropping rooms are required to be specially built for the purpose. Two types of cropping rooms are built suited to particular requirements – those required for seasonal growing and those for environmentally controlled year-round growing (Figure 14.6).

14.2.4.1 Seasonal Cropping Rooms

Seasonal cropping rooms are simple rooms with modifications for maintaining various growth parameters. These cropping rooms will have a cemented floor, cemented walls, cemented ceiling, or a false ceiling with an arrangement for forced air circulation inside. The seasonal cropping rooms are built of simple brick walls with a roof made of asbestos sheets and a false ceiling. The room is more or less made air tight to make the air handling system work effectively for obtaining necessary air changes during growing. No insulation is required for seasonal growing rooms as it will not allow heat dissipation from the room efficiently. These simple rooms are used for seasonal mushroom growing, coinciding various phases of growth with prevailing outside temperatures. No energy is used for heating/cooling of the rooms under seasonal growing conditions. The cropping rooms for seasonal growing can also be made with a thatched roof and a false polythene ceiling. The door is installed on one end and the exhaust vents on the opposite end of the door. The forced air circulation fan is installed on top of the door (Figure 14.7). The mushrooms are grown on beds made out of bamboo sticks and sarkanda stems (a plant abundantly growing as a weed on the north-western plains of India). These growing rooms can also be built as a low cost structure, steel pipe frame with heavy density polythene covering from the outside.

The real low cost-low technology growing houses built in rural areas are made with bamboo frame and walls, roof and door of sarkanda stems for air exchange in the room through

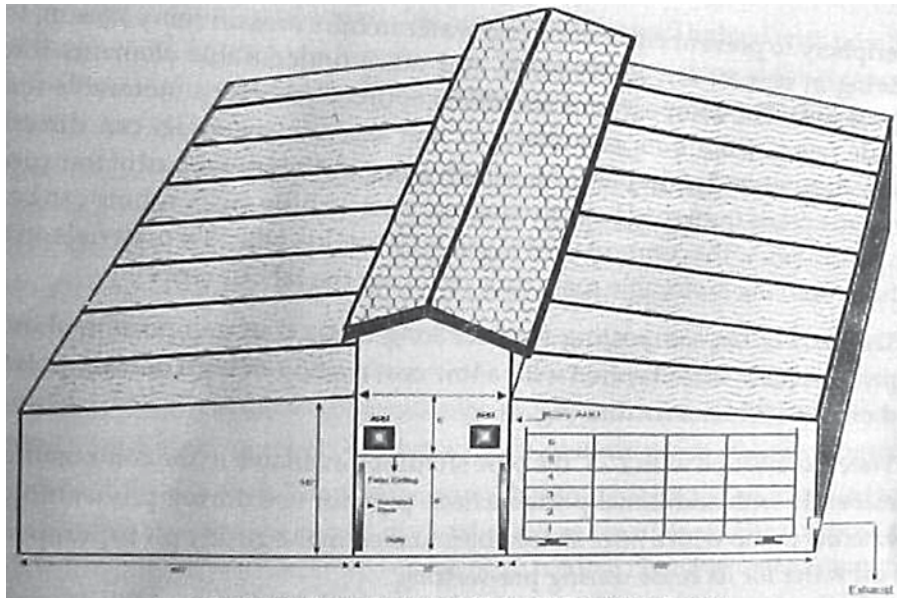

Figure 14.6 Front cross-sectional view of cropping rooms.

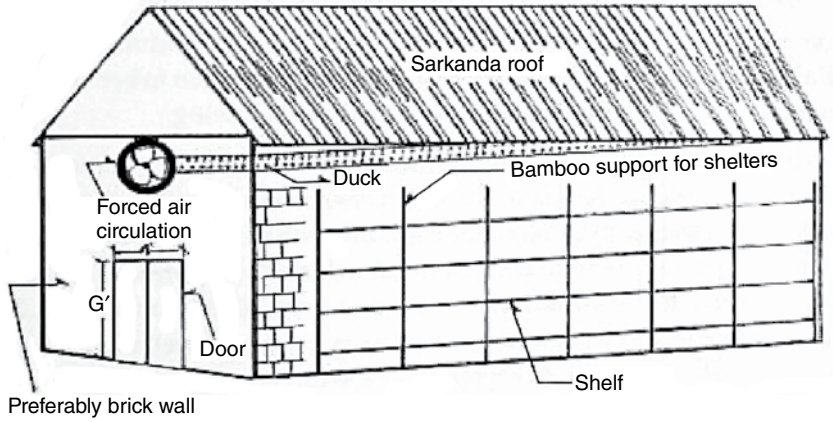

Figure 14.7 Low cost growing room.

porous stem walls all the time. But these houses are totally at the mercy of the climate and low winter temperatures interfere in the normal crop production. The mushroom houses made with bamboo frames and synthetic fiber cloth material, both inside and outside, with paddy straw insulation in between have also given good results for seasonal growing in hill conditions.

14.2.4.2 Structural Details Special to Low Cost Growing Rooms

14.2.4.2.1 Environmental Conditions for Seasonal Growing

For seasonal growing, all these requirements are met with by coinciding various stages of crop growth with the prevailing seasonal temperature outside. In hilly areas, the mushrooms can be

grown seasonally with minor adjustments raising 2–3 crops in a year. But for round the year cultivation in hills with greater productivity, environment control is essential to provide the required climate in hot and cold periods of the season. The seasonal crop can also be raised in plains close to hills in winter months with good profits. The hotter/tropical/sub-tropical areas will require air conditioning and environmental control all the time since temperatures in these regions range between 28–40°C year-round.

14.2.4.2.2 Forced Air Circulation

Forced air circulation is very essential for forcing in the oxygen and exhausting the CO_2 laden air during cropping, as the compost is microbiologically active and produces CO_2 all the time with consumption of oxygen. In controlled growing houses the forced air circulation is achieved by the use of an AHU that cools/heats, maintains RH, forces in fresh air, and exhausts the CO_2 laden air via back vents under positive pressure. Provision for forced air circulation can be made in seasonal growing houses by installing an exhaust fan on top of the door facing inwards and joined to a perforated polythene duct running along the entire length of the room. The walls and false ceiling should be air tight to make the forced air circulation system effective and workable. In low cost growing houses where thatched roof is used, a polythene false ceiling will be essential to create the sealing effect on top. The seasonal growing houses should not be insulated, as it will be difficult to maintain the right environment inside the room in the season congenial for crop growth. In extremely cold areas where lower temperatures are prevalent in some part of the season, brick walls with air gaps should be good enough to prevent condensation of water on the walls in the cropping rooms.

14.2.4.3 Environmentally Controlled Cropping Rooms

The environmentally controlled cropping rooms are built like hermetically sealed chambers where the air movement is controlled either manually or semi-automatically with mechanical control systems. These cropping rooms are appropriately insulated and the dimensions of a cropping room are determined by the amount of compost to be filled into the room. Rooms with greater length and narrower width give better results as far as air handling inside the room is concerned. A cropping room, with a capacity of filling compost from one bulk chamber, is considered advantageous as one bulk chamber load can straightaway be filled into one cropping room. Both bulk chamber and cropping rooms of 20–25 tons compost capacity are considered to be of operationally efficient size, as the operation of filling/emptying and spawning of 20–25 tons of compost can conveniently be done in one day when machines are not to be used. For this quantity of compost, cropping rooms with the following dimensions are in use in various parts of the world: 17 × 5 × 4 m; 18 × 7 × 4 m; 11 × 8 × 4 m; 18 × 7 × 3 m (55’ × 18’ × 12’; 60’ × 22’ × 12’; 35’ × 25’ × 13’; 60’ × 22’ × 10’; low cost structures) and 12 × 6 × 4 m (40’ × 20’ × 13’).

The cropping rooms with these dimensions are used with a shelf system inside (3–5 shelves), each room holding 20–25 tons of compost, the variation being adjusted more with varying the depth of compost layer. Polythene bags are also used for growing mushrooms in these growing houses, with an approximately similar capacity of compost holding (Figure 14.8).

The foundation of growing rooms should be laid on dry and firm ground. The floor is laid as explained for the bulk chamber. The walls will be made of one brick thickness (23 cm/9” thickness) and ceiling made of 10 cm (4”) thick RCC. The growing rooms will have a single insulated door and two vents for exhaust on the back wall at ground level. One opening is provided on top of the door for entry of the AHU delivery duct. The walls, ceiling, and floor should be insulated with 5 cm thick insulating material. The room should be made air tight and all leaks

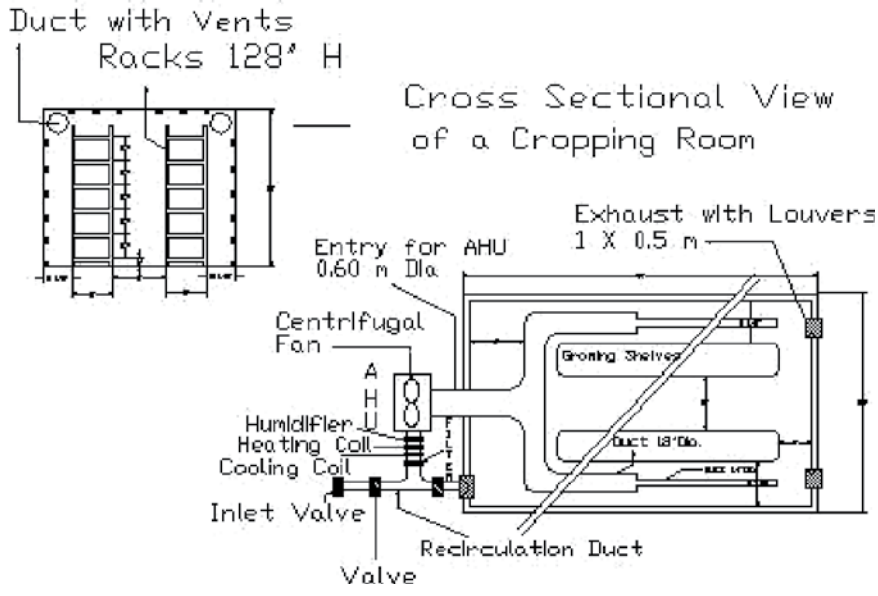

Figure 14.8 Internal layout of a cropping room (length 18 m/60′, width 7 m/22′, height 4.4 m/15′).

closed to prevent ingress of heat from outside. The cooling, heating, and forced air circulation in the growing room is done via an AHU installed for each cropping room individually. The floor and walls of the cropping rooms should have a smooth finish.

14.2.4.4 Structural Details Special to Cropping Rooms

14.2.4.4.1 Floor

The floor must be well laid out and should be strong enough to take the heavy load of metal racks to be installed inside for growing mushrooms. After digging start with a bed of sand 15–20 cm thick, followed by 5 cm thick concrete floor (1:3:6). The floor should then be insulated with insulating material 5 cm thick (sheets of thermocol or glasswool or polyurethane). The insulation should be protected by a PVC sheeting, below and above, against moisture. It is then covered with wire mesh and finally 5 cm thick concrete floor is laid on top, which is given a smooth finish. The floor should be with a slight slope toward the entry point for discharge of cleaning water and placement of a formalin trough for foot wash. The trough is connected near the wall to an exhaust drain to carry washings from the room. The water discharge hole is protected at this point to prevent leakage of air from the growing room.

14.2.4.4.2 Walls

The walls are made of brick 22.5 cm thick, which are given a smooth finish with cemented plaster. The insulation sheets are fixed on the walls (5 cm thick thermocol, glasswool/polyurethane), with the use of hot coal tar. Holes are drilled on four corners of the sheet/inside the cement wall for expansion fasteners which are fixed by screwing in the nail with 10–12 cm (4–5″) long steel wire tied on its head. The wire hangs out of the sheet to be used for tightening of wire net fixed on top of the insulation. The layer of cement plaster is then applied (2 cm) on top of this and given a smooth finish. Bituminous paint is applied on cement plaster as a vapor barrier. The painting can be avoided in cropping rooms if the cook out is not done by steam.

This wall will be good enough to give a k-value of 0.5–0.6 kcal/m^2h, even lower, and will facilitate proper control of climate inside the cropping room. The common wall will have insulation on one side only, or one inch of insulation applied on each face of the wall.

14.2.4.4.3 Roof

The roof is made of RCC (1:2:4) 12–15 cm thick. The inside is given a cement plaster finish for application of insulation (as explained for the wall). The roof on the outside is protected by tarring it on top, followed by 10 cm thick loose soil, 5 cm thick mud capping, and finally the tiles. This will protect the roof from weathering effects of rain and will ensure longer life of insulation and prevent seepage of moisture into the room in the rainy season. In hilly areas with a high rainfall index, slanting GI sheet roof over the insulated RCC roof will be excellent and in that case mud capping/tiling of the roof is not required.

14.2.4.4.4 Doors/Vents

The doors of the bulk chamber and the cropping room are made of wood or angle iron frame covered on the inside and outside with aluminum sheets/GI sheets with insulation of 5–7 cm in the middle. The doors will have a rubber gasket lined on the inner periphery so that the door becomes air tight when closed. The door will operate on hinges, with a strong locking latch for opening and closing of the door. The exhaust vents are normally made on the opposite end of the entry point, on the lower part of the wall at ground level. The exhaust vents are fitted with wire net, louvres, and insulated lids. The louvres allow the CO_2 laden air to exhaust under positive pressure created by the blower inside the AHU.

14.2.4.4.5 Lighting Arrangement

There should be provision for tube lights and a mobile strong light for inspection in each cropping room. The tube lights should be protected with waterproof housing. The tube lights should be fitted on all the walls vertically at various heights to facilitate lighting of all beds. There should be provision for a few electric points (5 and 15 A) for operation of water spraying equipment and CO_2 measuring instruments.

14.2.4.4.6 Water Connection and Sewers

One clean water pipe line (2.5/3 cm or 1/1.25") for delivering clean water for spraying should be provided in each room. Underground drainage line for carrying the washings from the room and wash basin discharge should be laid before construction of the building. This waste water line should be connected to the common sewer. In HD polythene cropping rooms, sunken traps on the floor for fresh water and drainage water are provided inside the growing house with each trap of 0.3 × 0.3 × 0.3 m (1' × 1' × 1') dimensions fitted with an iron lid on top. It is desirable to lay underground drainage in the central gallery in advance of erecting the structure for carrying away the waste water/washings from the cropping rooms.

14.2.4.4.7 Gallery

The gallery between the rows of cropping rooms should be wide (approximately 6 m/20') to allow efficient performance of various operations. The height of the gallery should be about 2.5 m (8') with a false ceiling, leaving another 1.5 m (5') above for the pipe line and space for AHUs.

14.2.4.4.8 Requirement of Environment Control and Forced Air Circulation in the Cropping Rooms

White button mushroom *Agaricus bisporus* requires 24°C for vegetative growth (spawn run) and 15–17°C for reproductive growth (mushroom production). The requirement of RH during

spawn run and case run is 90–95% and during cropping 80–85%. The third factor is fresh air requirement and removal of CO_2 plus heat from the cropping room. During spawn run there is very little requirement of fresh air and a higher concentration of CO_2 in the growing room is desirable for quick spawn run. During cropping fresh air requirement is tremendous and CO_2 has to be exhausted regularly. The CO_2 concentration during cropping, in general, should not be above 800–1000 ppm and this is adjusted as per the requirement of the strain of mushroom under cultivation. The amount of heat produced during spawn run, the first two flushes in the cropping room containing 20–25 tons of compost is about 4000–5000 kcal per hour, which has to be removed regularly by the cooling system during spawn run. Air changes, cooling/heating, RH, heat/CO_2 removal, and evaporation from the beds is constantly maneuvered inside the cropping room for getting a healthy crop of mushrooms. These factors are part of the environmental crop management and are as vital and important as quality of compost and spawn in mushroom growing. All these factors have to be maintained in a coordinated manner, as change in one factor affects the other. The enthalpy lines (Mollier diagram) available for a particular place becomes a guiding factor for environment/climate creation inside the cropping room (Griensven, 1988).

14.2.4.4.9 Climate Controls for Environmentally Controlled Growing

These factors, responsible for the production of a healthy crop of mushrooms, need to be simulated inside the cropping room in those areas where outside temperatures are not congenial for mushroom growth. The basic requirement for air conditioning/environment control in a growing house is the insulation of the cropping room to completely cut-off the external environment from the inside environment. The air entry is restricted and controlled as per the requirement of the cropping room with use of an AHU. The AHU contains the cooling coils, the heating coils, and one chamber for humidification, with a centrifugal blower fan in front to pull through the air into the room for circulation via air ducts placed in the room as per plan. The recommended air pressure of the blower fan should support 50 mm of water level at the entry point. The cooling coils are supplied with chilled water at 5–6°C from a chiller in the A/C room. The number of cooling coils to be used in the AHU will be determined by the compost load of the room and prevailing outside temperature and is always calculated for the maximum requirement during the hottest period. After its passage through the cooling coils the air is cooled to about 13–14°C before it is blown into a mist chamber for humidification. The air in the humidity chamber is brought to 100% RH at 13–14°C, and then blown into the ducts for circulation into the cropping room. By the time it reaches the crop bed, the temperature rises to about 16–17°C lowering the RH automatically to 85%. The air speed on the beds should not exceed 15cm per second, unless the air is amply humidified. The slow movement of air over the crop bed ensures slow evaporation of moisture from casing resulting in removal of CO_2 plus heat from the crop bed. The CO_2 gets mixed with the air and is finally blown out via the exhaust vents. This is the technique used during cropping for maintenance of correct climate inside the cropping room. The requirement of fresh air is adjusted to 30% outside fresh air during the first flush of the crop, 20% in the second and subsequent flushes. The rest of the air is recirculated from inside via recirculation ducts connected to the AHU. The fresh air/recirculated air quantities are mixed/controlled by adjustment of dampers at fresh and recirculated air entry points. The size of the damper is known and by measuring the air speed at entry point, the air quantity can be adjusted. The air displacement capacity of the AHU in a cropping room should be about 4500–5000 m^3/hour for a cropping room with 20–25 tons of compost. In temperate/cooler areas, the air can be forced in without pretreatment for cooling. In hotter regions cooling is required and for this the water is chilled (for use in the AHU) in the chiller (shell/tank) in the A/C room. The heat

ultimately is lost into the atmosphere via a cooling tower erected on top of the A/C room. For mushroom growing cooling is done indirectly by use of chilled water in the cooling coils in the AHU. Alternatively, the air can be preconditioned in the A/C room/air washers and then blown into all the cropping rooms, but this has a disadvantage that all rooms will have to be at one stage of cropping. Individual AHUs for each room will facilitate the use of different temperature ranges required at different stages of crop growth. The use of room air conditioners is not recommended for mushroom growing, as these will dry the cropping beds before it starts cooling. For heating of rooms, the heating coils in the AHU are supplied with steam from the boiler and the number of heating coils needed will again depend upon the prevailing outside temperature. The RH is created in the additional chamber in the AHU with fine steam jets located in the RH chamber for misting. The ducting in the cropping rooms is done with precise measurements, keeping in view the number of shelves in the room and the total bed area, with the amount of compost. The duct should be laid in such a way to ensure slow movement of air over the bed surface. This can be checked by burning incense sticks or small paper flags. That will ensure regular evaporation from the crop bed, which is essential for maintaining upward movement of nutrients in the compost bed.

14.3 Mushroom Cultivation Technology

14.3.1 Substrate Preparation/Composting

The commonly cultivated white button mushroom *Agaricus bisporus*/*Agaricus bitorquis* is one mushroom that requires more skill in substrate preparation, as it does not grow best on unfermented agro-wastes, as is the case with other cultivated mushrooms like *Pleurotus*. The substrate/compost for button mushrooms is prepared from agro-byproducts/wastes like cereal straw, sugarcane bagasse, barley/millet stalks, maize stalks, and other such materials. The cereal straws/other straws are used as base materials, which are supplemented/enriched with nitrogen rich animal manures like horse dung/poultry manures, and other such materials. The materials are wetted/blended in a definite proportion, subjected to solid state fermentation in two different phases and a selective medium prepared for the exclusive growth of mushroom mycelium.

14.3.2 Materials

The commonly available materials in the form of agro-wastes/byproducts are generally used as base materials for compost making. The most common are the cereal straws (wheat straw/paddy straw), sugarcane bagasse, maize stems, barley grass, hay, and other such materials. The commonly used animal manures are the N-rich poultry manure, horse dung, and other such materials, which are used as supplements/activators and total nitrogen content brought to 1.5% of the total dry weight (carbon content). For this we add some nitrogenous fertilizers and bring the C:N ratio to 35:1 at the start of the composting process. This is necessary as the mesophilic microbes that have to initiate the fermentation process and generation of heat in the compost require a basal C:N ratio of 35:1.

The availability of base materials like cereal straws and supplements like poultry manure guides a grower for determination of a formulation for compost making. No formulation is recommended as such but the formulation is developed after assessing the availability of raw materials/supplements in a particular area and the costs at which these materials are available.

14.3.2.1 Base Materials

Wheat straw	Paddy straw	Maize stems	
Jawar stems	Barley stems	Sugarcane bagasse	Hay

Animal manures

Horse manure	Mule dung	Poultry manure	Excreta from piggery

N-rich organic activators/supplements

Soybean meal	Cotton seed meal	Soybean cake
Cotton seed cake	Maize cob shells	Cotton seed hulls

N-rich fertilizers recommended

Ammonium sulfate	Calcium ammonium nitrate
Urea	Other N-fertilizers

14.3.2.2 Recommended Formulations for Composting in India (One Ton Base Material)

1. Wheat straw	- 1000 kg
Poultry manure (dry)	- 500 kg
Wheat bran	- 150 kg
Urea	- 16 kg
Gypsum	- 30 kg
Water	- 3500–4000 l
2. Wheat straw	- 500 kg
Paddy straw	- 500 kg
Poultry manure (dry)	- 500 kg
Wheat bran	- 150 kg
Urea	- 16 kg
Gypsum	- 30 kg
Water	- 3500–4000 l
3. Horse manure (wet)	- 800 kg
Wheat straw/paddy straw	- 500 kg
Poultry manure (dry)	- 300 kg
Urea	- 12 kg
Gypsum	- 35 kg
Water	- 3000–4000 l
4. Wheat straw	- 1000 kg
Wheat bran	- 200 kg
Urea	- 16 kg
Cotton seed cake	- 60 kg
Gypsum	- 30 kg
Water	- 3000–4000 l
5. Sugarcane bagasse	- 1000 kg
Paddy straw	- 300 kg
Poultry manure	- 800 kg
Urea	- 15 kg
Cotton seed cake	- 60 kg
Gypsum	- 35 kg
Water	- 4000 kg

(Author – formulation evolved)

14.3.2.3 Earlier Formulations Recommended in India (European Adoptions)

1. Wheat straw	- 1000 kg
Dry poultry manure	- 400 kg
Brewer's grain (wet)	- 72 kg
Urea	- 14.5 kg
Gypsum	- 30 kg
(Short method/commercial scale)	
2. Wheat straw	- 1 ton
CAN	- 30 kg
Super phosphate	- 10 kg
Urea	- 13.3 kg
Sulfate of potash	- 10 kg
Wheat bran	- 100 kg
Gypsum	- 100 kg
Molasses	- 16.6 kg
Nemagon	- 123 ml
Lindane	- 833 g
Long method/short method (Seth, 1976)	
3. Wheat straw	- 500 kg
Horse manure	- 1000 kg
Chicken manure	- 300 kg
Brewer's grain	- 60 kg
Urea	- 7 kg
Gypsum	- 30 kg
(Seth, 1976)	
4. Paddy straw	- 250 kg
Dried chopped maize stalks (3–4" long)	- 250 kg
Ammonium sulfate	- 15 kg
Super phosphate	- 15 kg
Urea	- 6 kg
Rice bran	- 80 kg
Gypsum	- 25 kg
Cal. carbonate	- 16 kg
(Sohi, 1974)	
5. Paddy straw	- 1000 kg
Mule dung	- 700 kg
Urea	- 20 kg
Potash	- 15 kg
Super phosphate	- 15 kg
Gypsum	- 50–60 kg
Wheat bran	- 60 kg

14.3.3 Composting Procedure

The entire composting process is accomplished in two phases, phase I and phase II. Phase I is again done in two parts, part 1 is pre-wetting, blending, and mixing; and part 2 is outdoor aerobic fermentation either in a stack outdoors on a composting platform or in a phase I aerated bunker.

14.3.3.1 Phase I

14.3.3.1.1 Pre-Wetting: Six-Day Process

- The raw materials are brought to the composting yard and straws are first wetted, either in a bog or by use of a sprinkler or water hose pipe on day (–6). The straw is thoroughly wetted and turned with forks/front loader so that all portions of the straw heap receive water. The wetted straw with water seeping out at the cement floor is left as such overnight/48 h to allow the moisture to be imbibed by individual straw cells in the heap. The water leached out during wetting is collected in a guddy pit, and reused the next day.
- The straw is turned and wetted again on day (-4) after 24/48 h (depending upon the type of straw used – paddy 24 hours, wheat – 48 hours). The water from the guddy pit is sprayed back onto the straw to utilize the nutrients leached out on the first day of wetting. The straw is again wetted and turned. This is again left standing in a wet heap for 24/48 h, to permit water absorption and dewaxing of the wheat straw cells.
- On day -2 the additional composting ingredients like wheat bran, fertilizers, cotton seed cake, and poultry manure are mixed with the wet straw to blend the composting material thoroughly, and water if required.
- The watering/blending are two important activities in compost making that are critical for manufacture of a good compost. At this stage composting ingredients should have 75% moisture.
- The partially fermenting composting ingredients are left at the pre-wetting yard for another 24/48 h to permit absorption of water to its maximum by the straw cells.
- On day 0, the composting materials are stacked after thorough mixing/blending/watering. A moisture level of around 75% is maintained at this stage. With the addition of poultry manure, the heating of the pile has a vertical takeoff, poultry manure works like rocket fuel and results in tremendous heat production in the first 24 h after stacking.

14.3.3.1.2 Outdoor Composting

The stack is made on the composting platform on day 0 or alternatively the ingredients are filled into a phase I aerated bunker, for aerobic fermentation. The pile outdoors is made 1.5 × 1.5 m (5' wide and 5' high), and the length will depend on the quantity of the material. For outdoor composting, wooden/steel boards are used to give the stack a smooth vertical wall on all sides for proper aeration of the compost pile (chimney effect – meaning that hot air escaping from the top will be replaced by fresh air going in from the sides).

- The stack is given the first, second, and third turnings on days 2, 4, and 6 for proper mixing/blending of ingredients and watering of portions that are not properly wetted. The turning facilitates uniform fermentation of ingredients/mixing and replenishment of fresh air into the stack for aerobic fermentation. Ensure that the temperature in the stack before each turning is around 70–75°C. Gypsum is mixed on the third turning when maximum ammonification has been achieved (0.4% ammonium at the third turning/at filling into phase II chamber). The material is filled into the chamber quickly to preserve heat and phase II operations resumed.
- Alternatively, an aerated bunker can be used in place of open stacks for phase I composting process. The composting ingredients after pre-wetting are filled into the aerated phase I bunker over the grated floor to the height of 1.8–2.4 m (6–8'). The material is left in the bunker for 48 h for high temperature fermentation. The blower of the aerated floor is operated for 5–6 min every hour to replenish oxygen in the composting ingredients and drive out foul gases. The oxygen availability is made a limiting factor and with restricted oxygenation of the composting materials, less foul gases are produced and emitted. That is why this method is

termed as ecofriendly. The composting ingredients are drawn out of the phase I bunker and put on the platform and after a few hours refilled into the phase I bunker again. This helps in mixing/blending the materials well, inspect the composting materials for production of fire fang/moisture content, and apply corrections if required. As a matter of principle, the watering/mixing should be perfectly done during pre-wet operations and the amount of water required should be blended into the material during the pre-wetting operations. The entire process of composting will go out of gear if this stage is not done religiously. The ingredients are allowed to stay in the bunker for another 48 hours and again drawn out the third time and poured onto the platform after mixing gypsum with them.

- The ingredients are monitored for temperature at each filling. The temperature in the core region goes to 70–75% or even up to 80°C, but the bottom/sides/top show lesser temperatures of around 60°C. It is in this region that useful thermopiles are left surviving to help in phase II operation later.
- The composting ingredients, after solid state uniform aerobic fermentation in phase I bunker for 6 days with emptying/refilling done after every 48 hours, are ready for filling into phase II (pasteurization chamber). The material is quickly loaded onto the grated floor of the pasteurization chamber to the height of 2 m, the chamber closed, and the blower switched on.

14.3.3.2 Phase II

14.3.3.2.1 Pasteurization/Conditioning

Phase II of compost making starts after completion of phase I. After filling into the bulk chamber/peak heating chamber, the material is maintained in the closed chamber with the blower fan switched on to equalize the temperature of compost/air below and above the compost. The heat is given out from the fermenting compost and this results in heating of air above and below the compost mass slowly. The compost is at a temperature of 45–50°C when filled into the chamber and slowly the air above shows a temperature rise followed by a rise in temperature in air in the plenum. The temperature is equalized in 6–8 hours or even 12 hours with the blower continuously on and fresh air inlets closed. After equalization of temperature, steam is injected to increase the temperature of the compost as quickly as possible to pasteurization range (57–59°C air temperature.), which may take 8–12 hours depending on the quality of compost in the chamber and capacity of the boiler. The time is noted when the air temperature above the compost and the plenum reaches 57–59°C. The temperature is held in this range for 8 hours for the pasteurization process. The compost temperature may also be in the range of 58–60°C but air temperature should not be allowed to go beyond 59°C. Use of steam is made intermittently, if required, to maintain the air temperature (57–59°C) in the desired range during pasteurization process. After completion of the pasteurization process, a fresh air vent is opened 20% to let in fresh air for aerobic high temperature fermentation of the compost. The compost temperature tends to fall slowly on opening of the fresh air vent and it is allowed to drop slowly to 48–53°C for the conditioning process to resume. The temperature of the compost is held in this range for 5–6 days till the ammonia smell is no longer discernible. After completion of conditioning, more fresh air is brought in by opening the vent to 50–100% and the compost is cooled down to 25°C before spawning. In tropical areas use of cooling coils is made to cool the compost temperature to 25°C as the ambient temperature in summer is around 40–45°C. During the process of conditioning the blower fan is kept on non-stop, the fresh air vent kept open at 20% fresh air with exhaust of gaseous air from inside through the exhaust outlet. Steam can be injected, if required, to keep the compost temperature in the desired range. During the process of conditioning the ammonia (free/bound) is converted to microbial proteins by thermophilic microbes (mostly fungi). Phase I is done to bring composting materials to the stage of maximum ammonium production, and phase II to facilitate the conversion of ammonia/ammonium

to microbial proteins in the compost, which is selectively utilized by mushroom mycelium later for mushroom production. The compost after completion of phase II of composting should be dark brown in color, dull, and non-greasy looking, with nitrogen content at 2.3–2.5%, moisture at 67 ± 1 %, pH in the range of 7–7.5 (even up to 8), and with no presence of off-odor/ammonia (to be detected by smell). If a dragger tube is available, ammonia content should be below 3 ppm.

14.3.3.2.2 Composting Procedure for Single Phase Composting without Steam Pasteurization

The long method of composting is done on an outdoor composting platform where composting ingredients are stacked on day 0 (after pre-wetting for 2–3 days), with the first turning given after 3–4 days of stacking when the stack temperature reaches 70–75°C, and subsequent turnings given on the outdoor platform after every 2–3 days (6–7 turnings in total), until the ammonia smell is eliminated. Gypsum is added on the third turning when ammonia production is at its maximum. After 6–7 turnings the compost becomes dull-dark brown in color, non-shiny, and with a pleasant/sweet compost odor. The compost temperature by the sixth or seventh turning comes down to about 45–50°C naturally. The compost is then allowed to cool to 25°C before spawning. For more information about composting production consult Chapter 6.

14.3.3.2.3 Spawning and Spawn Run

Spawning of the compost is done immediately after completion of the composting process and when the compost temperature has been brought down to 25°C. The grain spawn of *A. bisporus* is mixed with the compost under aseptic conditions and seeded compost filled into polythene bags or beds, compressed hard, and leveled. The mouth of the polythene bag is closed loosely, rather than folded, to prevent evaporation of moisture from the compost. If filled in trays/shelves, the seeded compost after compression/leveling is covered with a clean newspaper sheet. Bags can be filled from 0.3–0.5 m (12–20") depth, shelves can be filled up to 0.2–0.25 m (8–10") depth. The newspaper is wetted daily to prevent moisture loss from the bed. The desired quantity of spawn is directly mixed with the compost by the through-spawning method (0.5–0.7% of wet compost weight) and seeded compost filled into the bag or shelf or tray. Spawn can also be added to the compost by layer spawning method, top layer spawning, or dibbling all over the compost. All methods of spawning are at par, and it is the convenience of the grower that decides the method that he chooses for spawning. The spawned compost is placed inside a cropping room, maintained at a temperature of 23 ± 1°C (air temperature.), RH of 95%, and high CO_2 concentration (10,000–15,000 ppm strain dependent) for an effective spawn run. There is no requirement for fresh air during spawn run, and all vents are kept closed during the process of spawn running, which takes about 12–14 days. The entire compost mass turns light brown after spawn impregnation/spawn run. The grower must ensure that pure culture spawn, freshly prepared, is used for spawning, which is done under aseptic conditions. The spawning area, tools, hands should be sterilized with formalin before spawning is done. All the doors/windows in spawning area should be kept closed during the spawning operation.

14.3.3.2.4 Casing and Case Run

Casing layer is a layer of soil 3–4 cm thick applied on top of spawn run compost and is a prerequisite to fruiting in *Agaricus bisporus* cultivation.

Casing Materials

In the past sub-soil material or organic matter rich soils were used as casing in button mushroom cultivation. Presently peat is the most desirable casing material used worldwide with excellent mushroom yields and superior fruit body quality. There are several other alternative materials now recommended for use as casing materials in white button mushroom cultivation.

These are well decomposed FYM, well decomposed spent mushroom compost, composted coir pith (coir industry waste), decomposed powdered bark of some forest trees, paper industry waste, and so on. The casing materials should be soft, pliable, and capable of tremendous water holding capacity, capable of permitting maximum air exchange/ion exchange, and above all, be deficient in nutrient materials supporting vegetative growth of the fungus. The casing materials should have C and N in unavailable forms, otherwise there will be no fruit body formation. The casing material should be almost neutral in pH with low electrical conductivity (400–600 μ moh). Sand, burnt paddy husk, ash, and gobar-gas-compost are undesirable casing materials used by many growers in Indian in the absence of viable casing materials. Sugarcane-pressmud in combination with coir pith has also shown promise as a healthy casing material in India. Casing material should not be sieved but used as such with clumps, which permits more air spaces in casing. The casing surface should have small hills and valleys.

Casing Treatment

Before application, casing material is steam treated at 65–70°C for 6–8 hours. The casing material is wetted to 40–50% water holding capacity, filled into the casing pasteurization chamber and steam injected into the chamber till the temperature in the casing medium reaches 65–70°C. Hold the temperature in this range for 6–8 hours. The casing material is allowed to cool before application.

Alternatively, casing material is treated with formalin, using 1 liter of *formaldehyde 40* per 1 cubic meter (approx. 1000 kg) of casing material in concentrated form. Heap the wet casing on a cemented platform and apply formaldehyde to the wet casing directly at 1/1 m^3 and mix with a shovel. Cover the casing with a polythene sheet and seal the outer periphery by pouring sand/soil on the outside margin. Allow the casing to stand like this for 24–48 hours in sun for a fumigation effect. Formalin gas will be produced at temperatures of 25°C and above, which will kill all the living microbes, insect pests, and fungi, rendering the casing medium safe for use. Before use, the casing material should be exposed to sunlight, spreading it over with clean tools and permitting the formalin fumes to escape into the air for 2–3 days. Care should be taken to prevent re-infection of the casing materials. Store the treated casing material in a sterilized, clean room in polythene bags or synthetic cloth bags.

Casing Application

Apply the casing layer over leveled fully spawn run compost, 3–4 cm deep uniformly. Wet the spawn run compost by giving a light water spray, then apply casing. Use metallic rings 3–4 cm wide or wooden buttons 4–5 cm thick for application of uniform depth of casing layer. Water spraying should be done immediately after casing application to make the casing wet and bring it to maximum water holding capacity. Care should be taken that the water does not run into spawn run compost. It is best to apply water to casing in a few installments to bring it to maximum water holding capacity. For more information about casing consult Chapter 7.

Case Run and Pinhead Formation

Case run or impregnation of mycelium in casing in *A. bisporus* is done at a temperature of 24 ± 1°C, 95% RH, and CO_2 concentration upward of 7500 ppm (strain dependent). It takes about one week for complete case run at the above mentioned environmental parameters. There is no requirement for fresh air introduction during case run. The case run is considered complete when mycelium appears in the valleys of the casing layer. At this stage the environmental conditions are changed by lowering the temperature to 15–17°C (air), RH to 85%, and opening the fresh air ventilation to bring in oxygen and exhaust CO_2 to bring the CO_2 concentration in the room down to 800–1000 ppm (strain dependent). This change in environmental parameters

induces pinhead formation in 7–10 days (strain dependent) time. The pins develop into solid button sized mushrooms in another 3–4 days. The air in the cropping room is changed six times in 1 hour to maintain appropriate CO_2 concentration in a crop room, as CO_2 is at its peak during the first flush (actually peaks at case run).

14.4 Environmental Crop Management

During the entire cropping period, the air temperature of 15–17°C and 85% RH is maintained in the cropping room, with CO_2 concentration held at 800–1000 ppm. The bed temperature should always be 1–2°C higher than the air temperature to permit slow evaporation from casing, necessary for upward movement of nutrients in the compost for obtaining healthier flushes of mushrooms. A flush break lasts 4–5 days or even 7 days depending on the intensity of the flush. In between the flush breaks, no stray mushrooms should be left growing on the bed as it will delay the formation of the next flush. If possible, raise the air temperature by 2–3°C for 24–48 hours in between the flushes to accelerate formation of subsequent flushes. During first and second flush, six air changes are required per hour to get the desired oxygen – CO_2 concentration in the cropping room, and after the second flush only four air changes are required as lesser quantities of CO_2 are produced with the passage of cropping time. Use the CO_2 meter to maintain CO_2 concentration in the cropping room at desired levels. Air temperature, RH, and CO_2 concentration are three important and vital parameters to be strictly maintained during pinhead formation in the cropping room to obtain a good flush of mushrooms. Deficiency in one of these parameters during pinning will lead to reduced pinhead formation, thereby resulting in reduced mushroom yields. All the parameters are interdependent upon each other, and have to be maintained in the right range for optimal results. Possibly, that is the reason why computer controlled environment maintenance is considered superior as it results in significantly higher mushroom yields for the reasons explained previously. Computer control synchronizes the control of environmental parameters in the desired range for greater productivity.

Mushrooms are harvested when buttons are of 4–5 cm in diameter, tough, stout, and hard. Hold the mushroom between your forefingers and thumb, rotate it gently to disconnect it from the mycelium in the bed. Dress the mushroom by cutting off the soiled stem portion and collecting different grades of mushrooms in different baskets. Apply fresh casing at places where mushrooms have been removed. Add water at the rate the mushrooms have been harvested, that is for every kg of mushroom harvested add 1 liter of water after harvesting. Do not allow the casing to dry, as it will result in sealing of casing and mat formation.

14.4.1 Watering

The mushroom contains 90% water and that should give us an idea how important water is for crop raising. Mycelium gets water from compost during spawn run and compost + casing during case run and from casing during fruit body formation. The water level in casing can be maintained in two ways at the optimal level for growth of mushrooms. One is by regular water spraying when pins are pea sized and second is maintenance of RH at 85% during cropping, ensuring slow evaporation from crop beds for upward movement of nutrients in the compost. If one of the factors, water spraying/RH maintenance during cropping is disturbed, it affects crop productivity. Low RH in the room will encourage quick drying of casing, thereby affecting normal development of the fruit body. Low RH during cropping will result in drying of beds, lightweight mushrooms, discoloration of mushrooms, and crop losses. Drying of casing will seal the casing medium and result in formation of mat which becomes impervious to water,

and results in tremendous crop losses. Water has to be replenished in casing to accommodate the water loss from casing due to mushroom growth and evaporation into the room air. It is not desirable to have 100% RH in the cropping room during cropping as it will not permit slow evaporation from crop beds, thereby preventing the nutrient upward movement in the compost and loss of CO_2 + heat into the room air for removal by the AHU. This fact has always to be kept in mind while raising a crop of mushrooms and the importance of water realized. Bed moisture and RH are two different factors, directly dependent upon each other. Bed moisture loss by way of crop growth is desirable, as it will ensure harvest of healthy/solid mushrooms but this loss should be replenished immediately and water added to the bed equal to the weight of mushrooms harvested. That should be the rule of the thumb, as far as watering during cropping is concerned (1 kg mushrooms harvested/1 l of water added to casing). Avoid watering of beds at pin breaks. The casing should be wet enough when fresh air is brought in and room temperature lowered. That wetness should be sustained till pinheads become pea sized, and that is the stage when the bed will require additional watering to allow pea-sized pins to develop into button sized mushrooms. Water management in mushroom crop management is the most critical of factors, requiring experience and skilled application. Watering of beds requires quantification at each stage, and trained workers handling watering will ensure proper water management in the cropping room. To monitor the air and bed temperature in cropping beds in the cropping room, use two ordinary stem thermometers, placing one in the casing/compost bed and one hanging in the air nearby (a few cm apart). Ensure that bed temperature is always 1–2°C higher than air temperature, which confirms that the air circulating in the room is humidified enough to prevent heavy evaporation from crop beds. When the air temperature is greater than the bed temperature it indicates the air in the room is dry and is removing moisture plus heat from the crop bed, thus showing decreased bed temperature. This is an undesirable situation and will require quick remedial measures to prevent crop losses. The remedy is to increase RH to appropriate levels immediately, ensuring smooth crop production. Computer control of AHU for climate creation in the crop room ensures application of cropping parameters with precision during spawn run, case run, and cropping. Use clean water free from salts, heavy metals, and other impurities for watering of the crop beds. Water that is good enough for drinking/watering for vegetable/field crops should be good enough for mushroom cultivation. It is desirable to test the quality of water before mushroom growing is started at a particular site. Test the water for pH, salts, heavy metals like iron/lead and other undesirable residues. Do not use sewage water for watering of crop beds during mushroom cultivation. Avoid use of chemicals/pesticides during crop raising to harvest chemical free mushrooms. Fungi are very efficient in uptake of pesticides from the substrate, and use of pesticides/other harmful chemicals should be avoided as far as possible.

For pest control, selective use of pesticides is recommended under advice. Preventive measures for exclusion of pests during mushroom crop raising is the best method of pest control in mushroom cultivation. Once the infection is established in the crop beds, eradication by chemical control is a difficult task and not very successful in mushroom cultivation. Pesticide/chemicals leave heavy residues in the fruit body, as mushrooms have to be harvested every day after spray application, and hence should be avoided. Use of biocontrol agents/plant products for pest control should be resorted to, if necessary.

14.4.2 Harvesting and After Care

The mushrooms when fully mature/grown to the right biological stage (hard button size) are ready for harvesting. Button sized mushrooms 2–5 cm in cap diameter, with closed veil and hard pileus are ready for harvesting. Hold the mushroom between your forefinger and thumb,

rotate the mushroom clockwise/anticlockwise to disconnect it from mycelium in the casing. Cut the soiled stem portion and collect the cut mushroom in a basket grade-wise. Do not drop the stem cuttings on the floor or the bed, as these will invite undesirable organisms to develop on it, thereby starting a problem in the cropping room. Clean the beds/floor after harvesting. Pour fresh casing materials at places where mushrooms have been removed/harvested, then spray water over the crop bed to leave it in excellent condition for development of the next flush. Remove browned pins/mushrooms, if any, from the bed by hand and pour fresh casing at these places. If bunching is observed, address climate controls for creation of optimal environmental conditions during pinhead formation. If onion sized mushrooms/drum sticks are observed, correct air circulation in the room for effective CO_2 removal from crop beds. Lack of air movement over crop beds and accumulation of CO_2 creates this type of situation on the crop beds. Long stemmed mushrooms are again the outcome of CO_2 accumulation in the air around the crop canopy due to faulty air movement/air circulation in the cropping room. Mushrooms after harvest are separated into different grades, packed in PP bags/cardboard boxes and preferably chilled at 4°C for 6–8 hours before sending to the market. The pre-market chilling enhances the shelf-life of mushrooms. While harvesting, care should be taken to keep the pileus free of casing soil so that the mushroom is not stained. Washing of mushrooms is undesirable, especially washing with potassium metabisulfite solution to make these extra white for reasons of increased acceptability in the market. Unwashed mushrooms stay fresh for a longer period. Mushrooms should be handled carefully, and not bruised during the harvest operation. Bruising will turn the pileus dark/pink on exposure to air. While packaging mushrooms in PP bag, ensure that a small hole (0.2 mm) is made in each PP bag to prevent the development of aflatoxins in transit or storage. Button mushrooms can be stored at 4°C for a few days without any deterioration in quality but it is desirable to consume/market the fresh mushrooms immediately after harvest. Since button mushrooms have a very short shelf life, they cannot be stored for longer periods and hence will require to be processed for longer storage. Mushrooms are best preserved in brine solution after blanching, either in cans or jars. The processed mushrooms stay in good condition for over a period of 1 year if canned properly under aseptic conditions. It is possible to transport canned mushrooms in containers over longer distances without any deterioration in quality. But fresh mushrooms can be transported short distances only in refrigerated vans/by air to reach remunerative markets. Mushrooms can also be freeze-dried for export and freeze-dried mushrooms retain the original food value, flavor, color, and texture. But this method is expensive as the machinery has to be imported from an industrialized country at a very high cost. For more information about harvesting and processing of mushrooms consult Chapter 13.

14.5 Cultivated Edible Specialty Mushrooms

The word specialty mushroom has different meaning for different countries. In India, the specialty edible mushrooms are mushrooms that are not grown and available commonly. Any mushroom other than the popularly grown white button mushroom *Agaricus bisporus* falls under the specialty category, and these mushrooms are generally not available over the counter from supermarkets/vegetable vendors in India. The mushrooms in this group are the different species of oyster, Shiitake, black ear, shimeji, enoki, milky, straw mushrooms, and others. Similarly, specialty mushrooms in EU countries/USA and other countries in Europe/America are those other than the white button mushrooms *A. bisporous.* But in China and many Far-Eastern countries in Asia, most of our specialty mushrooms are commonly grown there as a home-grown crop, and are easily available everywhere in these countries over the counter,

Table 14.1 Edible specialty mushrooms/optimum temperature for cultivation.

Popular Name	Scientific Name	Optimum Temperature
gray oyster	*Pleurotus sajor-caju*	20–28°C
black oyster	*Pleurotus ostreatus*	18–22°C
white oyster	*Pleurotus florida*	20–28°C
pink oyster	*Pleurotus djamor*	20–26°C
yellow oyster	*Pleurotus citrinopleatus*	24–26°C
king oyster	*Pleurotus eryngii*	18–22°C
black ear mushrooms	*Auricularia polytricha*	24–26°C
buna shimeji mushroom	*Hypsizygus tessellatus*	18–22°C
Shiitake mushroom	*Lentinula edodes*	18–22°C
milky mushroom	*Calocybe indica*	28–32°C
paddy straw mushroom	*Volvariella volvacea*	30–35°C
enoki mushroom	*Flammulina velutipes*	10–15°C
portobello brown cups	*Agaricus bisporus*	18–20°C

both in fresh and dehydrated form. In China, the mushroom is the sixth important crop in the country as far as revenue generation for the nation is concerned. The button mushroom is a specialty mushroom for these countries, and is chiefly grown for export/distant markets in processed form.

Specialty mushrooms that were cultivated at the Mushroom Research Development and Training Centre (MRDTC), Delhi in India for commercial cultivation are listed in Table 14.1 with the optimum temperature requirement.

14.5.1 Gray Oyster Mushroom

Pleurotus sajor-caju [(Bano, Z (1974); Block, SS (1958); Chang, ST (1978), Jandaik, KL (1976), Zadrazil, D (1978)]. At pinhead formation, the pileus of the gray oyster mushroom is gray to dark-gray. The color of the pileus changes to light gray on maturity, with fan shaped fruit body and thick texture. The fruit bodies are weighty when fully grown, and the pileus diameter may extend up to 10 cm (4”). This oyster mushroom is very selectively grown in India under seasonal growing conditions at temperatures ranging between 20–28°C, but growth stops at air temperatures above 28°C. This mushroom performs excellently when grown under controlled environment conditions.

14.5.1.1 Substrate Materials

P. sajor-caju grows best on wheat straw substrate which is easily available in India at affordable prices. Other alternative materials that are used for substrate preparation are maize stems, maize cob shells, pseudo banana stems, and other agricultural recyclable wastes. These base materials are supplemented with N-rich supplements like wheat/rice bran at 10% dry weight of base material. These materials should be fresh and free from molds and with maximum 10–15% moisture content. The water used for wetting and watering should be at a pH of 7.

14.5.1.2 Substrate Preparation

The base material is wetted on the composting yard floor with a water hose and left overnight for water absorption by straw cells. The heap is made rectangular shaped, about 0.7–0.9 m (2.5–3') in height and turned the next day after 24 hours before filling into the pasteurization chamber. The base material is alternatively soaked in a water tank in gunny bags overnight. The material is brought out of the tank and heaped on the floor of the composting yard to drain off excess water. The material after wetting is filled into the pasteurization chamber for steam pasteurization at 65°C for 8 hours. The material is loaded directly onto the grated floor up to a height of 1.8 m (6'), steam injected to raise the temperature of the material to 65°C, and the blower fan switched on. The material is held at this temperature for 8 hours. Alternatively, the wetted material is filled into the perforated plastic crates and loaded into the chamber for pasteurization of the material. The material after pasteurization is cooled to 24–25°C overnight, and then spawned in the aseptic spawning area located on the other side of bulk chamber. Rice bran/wheat bran is filled into the perforated crates and pasteurized along with the substrate at the same temperature, which is mixed with the substrate at the time of spawning.

14.5.1.3 Spawning and Spawn Run

The spawning is done inside the glazed spawning area located on the cleaner end of the bulk chamber. The spawning is done at 2% spawn rate (2 kg per 100 kg wet substrate). The pasteurized rice/wheat bran is also mixed during spawning operation with the base material. The spawning is done by the "through-spawning method," and seeded substrate filled into the perforated polythene bags, pressed hard to remove air pockets. The bags are tied on the top, then shifted to the spawn incubation room and maintained at 24–25°C and 90% RH. The spawn run takes about 12–14 days at 24 ±1°C. The bags are allowed to become white on the outside for better results, and at that stage the bags are shifted to cropping room.

14.5.1.4 Opening of the Bags for Cropping

The polythene bags are cut and discarded, and spawn run substrate bundles placed on the racks in the cropping room. The cropping room is maintained at a temperature of 17–19°C, RH of 85–90%, and CO_2 content of 800 ppm. The climate is automatically simulated to these levels, and the pinhead/primordial formation takes place in another 4–5 days. The primordia/pinheads grow into harvestable mushrooms in another 3–5 days (Figure 14.9a).

14.5.1.5 Cropping and Crop Management

The color of the pileus is dark-gray to gray in color, which turns lighter on maturity. During the cropping period from pinhead to maturation, a light source of about 400 lux is made available to the developing mushrooms as per requirement of the mushroom. The mushrooms are allowed to grow to full biological maturity, say 7.5–10 cm (3–4") of pileus diameter. The cropping room is maintained at these growing parameters throughout the cropping phase. The filters installed on the AHU require periodic weekly washing to remove the film of *Pleurotus* spores on the filters, which would hamper the air flow into the cropping room. The cropping is done for a maximum of 2–3 weeks, and the spent substrate discarded at the end of cropping. On average 20–22 kg of excellent quality fresh mushrooms are harvested from 100 kg wet substrate. Gray oyster mushrooms, *P. sajor-caju* are large at harvesting time with a hard/thick pileus, spreading out like a fan. Pileus diameter of 7.5–10 cm (3–4") is considered right for harvesting, and is accepted by the consumer with a smile. During cropping regular water sprayings are given to the crop beds. No chemicals/pesticides are used during the entire process of cropping. For more information about oyster mushroom cultivation consult Chapter 16.

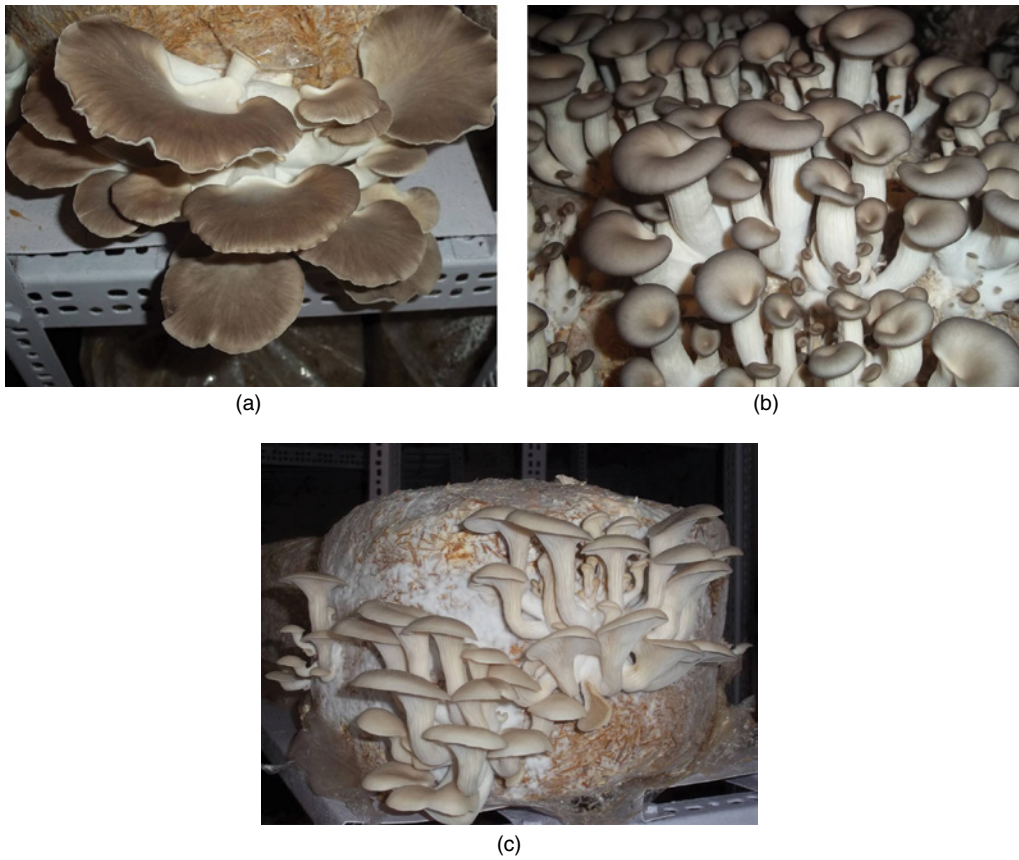

Figure 14.9 Gray oyster mushroom (a), black oyster mushroom (b), and white oyster mushroom (c).

14.5.1.6 Post-Harvest Handling and Marketing in India

The mushrooms are harvested at the right biological stage of the fruit body; that is when it is fully grown with pileus tight and curved inwards on the edge. The gills are decurrent and stipe is thin and short. The bulk of the fruit body is constituted by the pileus itself. This mushroom is very attractive to look at and fleshy in appearance. The mushrooms are plucked singly or in bunches from the substrate, collected in a basket, graded and packed in polythene bags/paper bags for the market. The polythene bags are provided with a small hole for air exchange to prevent the development of aflatoxins on the fruit body. The mushrooms are chilled inside a cold storage at 2–4°C for 4–6 hours before dispatch to market. These mushrooms are sold fresh in niche markets at attractive prices.

14.5.2 Black Oyster Mushroom

Pleurotus ostreatus [Block, SS (1958); Chang, ST (1978); Dhar, BL (1978), Zadrazil, D (1978)] is the black oyster mushroom, as the pileus is black in color at the time of primordium/pinhead formation. The entire process of its cultivation is similar to *P. sajor-caju,* except that this mushroom grows in vertically long bunches, with each bunch growing in acropetal order (lower mushrooms younger/smaller, upper mushrooms larger). This mushroom is also called the Hiratake mushroom in Japan, and it loves the lower temperature range of 18–22°C. It is a prolific yielder, and yields 20–25% mushrooms of wet weight of substrate

over a cropping period of 3–4 weeks. The mushroom shows a distinct black color when young, which turns lighter on maturity. This mushroom is harvested in bunches, and packed/marketed like *P. sajor-caju.* It is in higher demand because of its velvety look/texture and excellent aroma. The diameter of the pileus of this mushroom is 5–7.5 cm (2–3"), but mushroom length comprising of pileus and stipe is about 7.5–10 cm (3–4"). The entire length of this mushroom is fleshy in texture, with decurrent gills on the lower side. The fruit body looks like a horse shoe. This mushroom has preferred marketability value, and is readily accepted in the market by consumers and the executive chefs of top hotels for its excellent taste (Figure 14.9b).

14.5.3 White Oyster Mushroom

Pleurotus florida [(Bano, Z (1958), Sohi, HS (1974), Zadrazil, D (1978)] the white oyster mushroom is white in color from primordium/pinhead formation to maturity, and this mushroom also grows in bunches. It is grown in a similar fashion to the previous two types. The pileus of this mushroom has thin margins, is smooth, and pileus thickness is lower compared to *P. ostreatus* and *P. sajor-caju.* The mushroom looks like a white disk, growing on a thick stipe with decurrent gills extending to the base of the stipe, unlike *P. ostreatus*/*P. sajor-caju*. This mushroom grows excellently in the 18–22°C temperature range but can grow up to 28°C. White oyster looks graciously white, with delicate flesh which is turgid in texture. The marketability of this mushroom is quite high with increased demand in the niche market in India. It is a real gourmet mushroom, with high culinary value and an intense mushroom aroma when used fresh (Figure 14.9c).

14.5.4 Pink Oyster Mushroom

Pleurotus djamor [Sohi, HS (1974)] the pink oyster mushroom looks gracious on the bed and yields profusely. The cultivation process for this mushroom is similar as described for *P. sajor-caju,* excepting that this mushroom requires limited water spraying during its cropping. The mushroom pileus is thinner as compared to the previous three species, leathery in texture, and looks like a pink queen on the beds. The pileus is up to 7.5–10 cm (3–4") in diameter, with little or no stipe, and pileus thickness is 3–4 mm at the outer edges. The outer border at the top of the pileus is pink; the gills on the lower side are pinkish too. This mushroom is not fleshy as compared to the previously described three species. The marketability of this mushroom is excellent and it sells at attractive prices in the niche market in India. It fruits profusely at lower air temperatures of 18–20°C in the cropping room (Figure 14.10a).

14.5.5 Yellow Oyster Mushroom

Pleurotus citrinopleatus [Sohi, HS (1974)] is a beautiful yellow/orange colored oyster mushroom, growing in large bunches on the substrate, and looks very attractive on the bed and grows in typical acropetal order. Its fruit body is large (5–10 cm/2–4" diameter) and velvety to touch, fleshy, and yellow to yellow orange in color. The cultivation process for this mushroom is similar as described for *P. sajor-caju.* The mushrooms have a long stipe with decurrent gills. This mushroom grows profusely on pasteurized wheat straw substrate supplemented with rice/wheat bran. The mushrooms are yellow or light orange colored when young but color turns lighter on maturity. This mushroom also requires light for fruit body development. It yields 18–22 kg fresh mushrooms in 3–4 weeks of cropping. It prefers a temperature a shade higher than other oysters like pink, black, and gray, and is closer in temperature preference to white oyster (Figure 14.10b).

(a) (b)

Figure 14.10 Pink oyster mushroom (a) and yellow oyster mushroom (b). (*See color plate section for the color representation of this figure.)*

14.5.6 King Oyster Mushroom

Pleurotus eryngii [Peng, JT (2000)] is really the king among oyster mushrooms, not from a size point of view alone but for its elegance in taste and aroma. This mushroom is also known by the name of Kabul Dhingri in India, and in the past it was harvested from natural habitats in Afghanistan and sold in India after dehydration at very attractive prices. This mushroom is now cultivated in cropping rooms all over the world, both under seasonal growing conditions and controlled environments. The cultivation technology is described in detail as follows.

14.5.6.1 Substrate Materials

The commonly used substrate material for cultivating this mushroom is sawdust, supplemented with 10–30% wheat/rice bran. Wheat straw substrate supplemented with rice/wheat bran also supports the growth of this mushroom, but the best quality mushrooms grow on sawdust substrate supplemented with rice/wheat bran.

14.5.6.2 Substrate Preparation

Substrate sawdust is supplemented with 30% rice bran (w/w dry) and wetted on the composting platform, using water with pH 7. Gypsum/calcium carbonate is mixed with the substrate for pH adjustment only (3% gypsum, 1% calcium carbonate). The substrate material after wetting is filled into the polypropylene bags of 1 kg capacity, material pressed hard into the bag by hand, and a hole made with a wooden stick in the center for inoculum. The plastic neck is fixed on the open end of the polypropylene bag and the bags then cotton plugged and sterilized at 15 psi for 90 minutes. Wheat straw substrate is pasteurized at 65°C with steam, but it does not support good spawn run. When wheat straw substrate is sterilized at 15 psi for 90 minutes as for sawdust, it supports good spawn run and fruits well, but yields are lower.

14.5.6.3 Spawning and Spawn Run

The bags are allowed to cool inside the autoclave and inoculated the next day on the laminar flow deck using freshly prepared grain spawn of *P. eryngii*. About 40–50 g of spawn (4–5 %) is poured into the sterilized bag through the neck, and grain spawn pushed into the hole with a

sterile glass rod, and cotton plug restored in the neck. The bags are then shifted to the incubation room and maintained at a temperature of 24–25°C. The spawn run takes 12–15 days for complete spawn impregnation of the substrate.

14.5.6.4 Opening of Bags for Fruiting

The bags are opened after complete spawn run, by cutting off the extra polypropylene near the upper edge of the substrate. The old spawn is scraped off and discarded, and water sprinkled over the substrate to keep it moist. The bags are then shifted to the cropping room, maintained at 17–19°C temperature, 85–90% RH, and 800 ppm CO_2 concentration. The pinheads start appearing on the top surface in 9–10 days, which grow into harvestable mushrooms in another 3–4 days. The King oyster loves cold temperatures and production stops at temperatures above 24°C (Figure 14.11).

14.5.6.4.1 Cropping and Crop Management

The bags are given water spraying daily to maintain the substrate in moist conditions. The mushrooms grow into large fruit bodies with brownish streaks on the pileus giving an attractive look. The pileus stays curved inwards on the outer edge, the texture is tough, and the fruit body is quite fleshy, with decurrent gills extended over the stipe. The stipe is quite thick and forms the bulk of the fruit body. The bags are maintained in the cropping room at 17–19°C constantly with 85% RH and 800 ppm CO_2 concentration. For more information about *P. eryngii* cultivation, consult Chapter 15.

14.5.6.4.2 Post-Harvest Handling

The fruit bodies are harvested when these are 12.7–15.2 cm (5–6") tall, with an average weight of about 100–250 g. These mushrooms grow large and need to be placed in larger containers/bags for marketing. King oysters have a superior shelf life and can withstand short distance transportation. They are marketed in India in a niche market as a specialty mushroom of high value. The average consumer of mushrooms in India does not know much about this mushroom, and it does not sell fresh over the counter. It is grown in China/Korea/Japan and other Far-Eastern countries for export, and large quantities of king oyster mushroom are marketed fresh/in dried form in India, Europe, USA, Canada, and other countries. In China, this mushroom is a home grown crop and large numbers of mushroom growers cultivate it on a commercial scale for local consumption and export to neighboring countries.

Figure 14.11 King oyster mushroom.

Figure 14.12 Crop of black ear mushroom.

14.5.7 Black Ear Mushroom

Auricularia polytricha [Chang, ST (1978)], black ear mushroom cultivation technology is similar as discussed for king oyster mushroom. It grows on both wheat straw/sawdust substrates, but sawdust substrate shows superior results. The spawn run is done at 24–25°C and 95% RH and the spawn run can be accomplished in 2 weeks. The bags are cut open/given slits on the sides and bundles placed on racks/hung in the cropping room and maintained at 23–24°C, with RH of 85%. The fruit bodies appear in 8–10 days, which grow into harvestable mushrooms in another 3–4 days. The fresh mushrooms are leathery to touch, thin, membranous, dark brown in color, and turn black on drying. The spawn for this mushroom has to be prepared from freshly prepared mother spawn raised from fresh fruit body cultures. Preserving mycelial cultures in the refrigerator is not recommended for this mushroom. It is best cooked after boiling in fresh water, water discarded (Figure 14.12).

14.5.8 Buna Shimeji Mushroom

Hypsizygus tessellatus, the buna shimeji mushroom is grown on sterilized sawdust substrate, and its fruiting is done at 17–19°C. This mushroom is a slow grower and likes cooler temperatures. Sawdust supplemented with 10% rice bran is used for its cultivation after sterilization at 15 °C, taking more than 30 days. The cropping is done at 17–20°C and RH of 85%. The fruit bodies appear in 8–10 days after bag opening and scraping. The fruit body is smaller in size, with a light wood color on the pileus (Figure 14.13). The stipe is long and pileus about 2.5–3 cm (1–1.5") in diameter, with a pileus thickness of 2–3 mm. The mushroom yield harvested is about 15 kg from 100 kg wet substrate in 4 weeks of cropping. There is limited demand for this mushroom as it tastes rancid and is chiefly used for garnishing soups. This mushroom has a brownish cap and a long stipe, with the pileus very delicate and umbrella shaped. It grows best at cooler temperatures.

Figure 14.13 Buna shimeji mushroom.

14.5.9 Shiitake Mushroom

Lentinula edodes [Royse, DJ (2005)], the prized shiitake mushroom grows on wheat straw as well as on sawdust substrates. But the fruiting is superior on a sawdust substrate. Sawdust supplemented with 10% rice/wheat bran and a small quantity of gypsum (about 3% dry w/w) is added before the substrate is sterilized at 15 psi for 90 minutes. The substrate is spawned on cooling on laminar flow at 2–4% spawning rate, using grain spawn. This mushroom grows in its vegetative state in two phases, one is the spawn run and the second is the browning process. The vegetative growth/spawn run is done at 24±1°C, which takes about 30–35 days. This is followed by the browning process at lower temperatures of 17–18°C for 25–28 days before it starts cropping. The browning process is started after the complete spawn run. The top end of the polypropylene bag is cut open and the bag inverted on the shelf bed on a wet surface for a few days. The substrate starts turning brown, which is given a cold water dip treatment after completion of the browning process for 6–8 hours at a temperature of 10–15°C. This induces quick fruiting. Browning is the critical phase of vegetative growth and no fruiting will occur if the browning process is not completed effectively as described before. For more information about *L. edodes* cultivation, consult Chapter 15.

14.5.10 Milky Mushroom

Calocybe indica [Doshi, A (1995)], the milky mushroom is snow white in color with a long stipe and a small pileus. The stipe is fleshy and constitutes the bulk of the mushroom. This mushroom is grown on wheat straw after steam pasteurization at 65°C for 8 hours, in polythene bags. The spawning rate is 5% of the wet weight of substrate. The bags after spawning are maintained in a cropping room at 28°C. The spawn run takes about 12–14 days. The bags are opened after a complete spawn run and beds cased with steam pasteurized casing material consisting of a mixture of coir pith + FYM + ordinary soil (equal parts w/w). The casing thickness used is 5 cm (2") and after casing application water spraying is done regularly to maintain casing in a

(a) (b)

Figure 14.14 Milky mushroom (a) and paddy straw mushroom (b).

wet state. Case run takes 12–14 days at 28°C. After case run, the room temperature is lowered to 25–26°C and the crop appears in another 7–10 days after complete case run. The primordia develop into fully grown harvestable mushrooms in another 3–4 days (Figure 14.14). The mushrooms attain the height of 10–15 cm (4–6") at harvest time. One kg of mushroom will have 10–12 fruiting bodies. This mushroom has a superior shelf life, and can be transported short distances for marketing without damage to its quality.

14.5.11 Paddy Straw Mushroom

Volvariella volvacea [Chang, ST (1978)], the Chinese mushroom/tropical paddy straw mushroom is the most delicious and tastiest mushroom among the edible group, with added aroma. But its shortcoming is its poor shelf life. It has to be consumed within hours of its harvest, or dehydrated. This mushroom is grown on paddy straw bundles at the center. Each bundle weighs about 500 g (dry weight) with 0.6 m (2') length. The paddy straw bundles are wetted overnight in a water tank. The bundles are removed from the tank, left for a few hours at the composting platform to drain off extra water. The bundles are then steam pasteurized at 65°C for 6 hours. On cooling the bundles are arranged into a bed over a perforated shelf inside a cropping room. Each bed is about 0.6 m long and 0.6 m (2') wide, consisting of 5–6 layers. The bed is spawned in each layer on the periphery, sprinkling some gram powder-200 g per bed to provide nutrition. Spawning is done at the rate of 3% wet weight of substrate, using grain spawn. The bed is completely covered with a polythene sheet and maintained at a temperature of 30–35°C with 100% RH. The spawn run is complete in 4–5 days, and on complete spawn run, the polythene cover is removed to allow entry of fresh air for primordium/pinhead development. The pinheads develop in another 3–4 days, which grow into harvestable fruit bodies in another 1–2 days (Figure 14.14). The mushroom is harvested at egg stage, up to 5 cm (2") in diameter, not allowing it to open like an umbrella. The mushrooms are packed in polythene for market. This mushroom is not recommended to be refrigerated, but can be stored at 15°C for a few hours.

14.5.12 Enoki Mushroom

Flammulina velutipes is the most valued delicacy in mushrooms in the world. This mushroom is again cold loving and is grown best on sawdust and rice bran mixture after steam

pasteurization of the substrate in polypropylene bags. The spawn run is done at 24°C and takes about 3 weeks. Bags are opened after complete spawn run and maintained at lower temperature of 8–10°C for cropping. This mushroom is white to light golden colored with small pileus and a long stipe. They are grown in bottles and the mushrooms are made to grow out of the mouth of the bottles. They are very good in taste and aroma, and are marketed in paper rolls put at the bottle neck when the mushrooms start growing out of the mouth of the bottle. For more information about *F. velutipes* cultivation, consult Chapter 15.

14.5.13 Portobello Brown

Agaricus bisporus brown is the portobello brown mushroom (Figure 14.15), very popular in the UK and other West European countries as a cup mushroom. It is the brown strain of button mushroom, a non-hybrid button mushroom strain which is grown on the same lines as the commonly grown button mushroom, but with the difference that this mushroom is grown to a full size of cap diameter of 10–12.7 cm (4–5") with the cap still curved inwards with tight texture. It grows to a full cap size in normal cropping days after pinhead formation, with temperature and RH in the desired range.

14.5.13.1 Raw Materials Used

The raw materials used during the entire cultivation cycle are organic based. No chemical fertilizer/pesticides are used at any stage of cultivation. The compost formulation is standardized by using carbon and nitrogen sources of organic origin. The C:N ratio is balanced by supplementing the base materials with organic based animal manures, wheat bran, brewer's grain, cotton seed cake, and other such materials. Compost and casing are steam treated for pasteurization and no chemicals are added any stage of its preparation, except gypsum (calcium sulfate), which is basically used for flocculating/coagulating the colloids in the compost and to remedy pH.

Composting formulations:

Wheat straw	- 1000 kg
Poultry manure	- 800 kg
Wheat bran	- 250 kg
Cotton seed cake	- 150 kg
Brewer's grain (wet)	- 400 kg
Gypsum	- 35 kg
Water	- 4000–4500 l
N-content at start	- 1.57

14.5.13.2 Composting Procedure

Phase I	***Phase II***
Pre-wetting	Outdoor composting, pasteurization and conditioning

-6, -4, -2 day 0, 2, 4, 6, 7 or 8 (fill) until ammonia elimination

14.5.13.2.1 Phase I

The turning schedules are more temperature dependent, ensuring temperature of 75°C±2 before each turning. The moisture content on day 0 is at 75%, indicated by slow flow of leaching black fluids from the base of the stack before the first and second turning. The moisture content is automatically reduced to about 70–72% indicated by leachates at the bottom present in minute quantities, but not flowing away as on the first day. Gypsum is added on the third

turning when ammonia production is at its maximum. The contents are filled into the chamber one or two days after mixing gypsum.

14.5.13.2.2 Phase II

Phase II is done inside the bulk pasteurized chamber, following standard procedure. Equalization of temperature is done with vents closed and steam injected immediately on temperature equalization to raise the air temperature to 57–58°C and held for 8 hours for pasteurization. After completing the pasteurization fresh air is introduced (20–25%), which brings the temperature down to around 50°C. The conditioning is done at air temperature of 48–53°C for 5 days till ammonia is eliminated. Ammonia content is checked with a dragger tube, which is below 3 ppm. The compost is tested for pH, moisture, N-content, dry matter, smell, and other quality attributes, before spawning.

14.5.13.3 Spawning and Spawn Run

Spawning is done at a spawning rate of 0.5% of wet weight of compost, which means adding 500 gm spawn to 1 ton compost, and spawning done by the through-spawning method. The spawned compost is filled into polythene bags of 10 kg capacity, compacted and mouth loosely closed (folded in). The bags are maintained for spawn run at 24°C (air temperature), high CO_2 concentration (vents closed), and 95% RH. The complete spawn run takes 2 weeks, when compost in all bags is fully colonized by mushroom mycelium. Spawn strain used is the brown strain of *Agaricus bisporus.* Spawning is done in a sterile area.

14.5.13.4 Casing and Case Run

Casing materials used are a mixture of:

1) FYM (2 years old)
2) Coir pith – well decomposed.

The casing materials are steam treated at 65°C for 8 hours before application. The casing is done in sterile area. Uniform layer of 2.5 cm (1") thick casing layer is applied over fully spawn run compost, using 2.5 cm (1") thick wooden buttons.

(a) (b)

Figure 14.15 Cultivation of portobello brown (a) and portobello with button mushroom, note the difference in color and size (b).

The compost surface is leveled before casing application. Water is sprayed over the casing layer in small quantities to keep the casing layer wet. Case run is done at the same growing parameters as spawn run, 24°C air temperature, 95% RH, and high CO_2 concentration (vents closed/no fresh air introduced). Case run is completed in 1 week.

14.5.13.5 Airing/Pinhead Formation/Cropping

The ventilation is opened after complete case run, bringing in about 30% fresh air and exhausting CO_2. The temperature is simultaneously lowered to 15–17°C (air temperature), RH of 85% (steam introduction), and CO_2 concentration to 800 ppm. The air temperature is maintained in the range of 15–17°C throughout with RH of 85%, and reduced CO_2 concentration (continuous exhaust of CO_2 laden air and introduction of 20–30% fresh air). It is ensured that bed temperature stays 1–2°C higher than the air temperature during the entire cropping period. Pinning is observed 8 days after airing and pins develop into harvestable mushrooms in 3–4 days. Luxurious mushroom growth all over the cased bed is observed with each flush lasting 5–6 days.

14.5.13.6 Harvesting

Average fresh mushroom yield of 20–22 kg is harvested in 4 weeks of cropping from 100 kg compost, with the bulk of crop yield obtained in the first 3 weeks of cropping. Mushrooms are harvested with care, and casing soil applied at places where mushrooms are picked. Water spraying is given after the crop harvest.

Mushrooms are harvested when the cap diameter is 7.5–10 cm (3–4”), cap edge curved inwards, and cap tough and tight in texture. Strict hygiene is maintained during cropping to prevent any source of inoculum gaining entry into the cropping room.

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15

Cultivation of Mushrooms in Plastic Bottles and Small Bags

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15.1 Introduction

Glass bottle cultivation of *Lentinula edodes* (Shiitake) and *Flammulina velutipes* (Enokitake) using sawdust was developed around 1928 by a Japanese *Agaricus* grower, H. Morimoto in Kyoto (Nakamura 1983). Subsequently, G. Hasegawa established a method of cultivation of *F. velutipes* on sawdust substrate in glass bottles. In the mid-1930s, commercial cultivation of *F. velutipes* using glass bottles was begun by farmers in northern Nagano, Japan. Though *F. velutipes* production increased in Nagano in the 1960s, farmers suffered heavy damage at the hand of the 1964 Niigata earthquake when glass bottles shattered. Consequently, glass bottles were replaced with plastic bottles.

Bottle cultivation of *L. edodes* began in Fuzhou, China and small plastic bag cultivation of this fungus developed in Taiwan around 1967 (Chang and Miles, 2004). In Japan, small plastic bag cultivation of *L. edodes* and *Grifola frondosa* (Maitake) was started commercially in the early 1980s and around 1975, respectively.

In 1997, button mushrooms (*Agaricus bisporus*) accounted for 32% of world mushroom production (Chang and Miles, 2004). By 2013, it is thought that *A. bisporus* production had reduced to a mere 13%. This eclipse was the result of a remarkable increase of "specialty mushroom" production in China. The main specialty genera cultivated in Asian countries include *Lentinula*, *Pleurotus*, *Flammulina, Hypsizygus*, *Grifola*, *Auricularia*, *Pholiota*, *Volvariella*, *Tremella*, and *Hericium*.

It is estimated that around 80% of world mushroom production in 2013 was cultivated in small plastic bags, and 4% of the production was cultivated in plastic bottles. Ninety-two percent of the world production of specialty mushrooms (excludes *Agaricus*) was cultivated using small bags (including use of non-sterilized substrate).

In Japan, 93% of edible mushrooms are cultivated in plastic bottles and bags. Small bag cultivation is the most popular method in Southeast Asian countries. In recent years, large-scale production of *F. velutipes*, *Hypsizygus marmoreus* (Buna-shimeji), and *Pleurotus eryngii* (King Oyster) using plastic bottles increased in China, Korea, and Thailand. Most recently, large-scale bottle production of *F. velutipes*, *P. eryngii*, and *H. marmoreus* by Japanese companies has begun in China, Taiwan, and Malaysia. Significant bag production of *G. frondosa* by a Japanese company has also begun in China.

This chapter covers the cultivation of specialty mushrooms under controlled environments using plastic bottles and small bags containing axenic substrate.

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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15.2 Characteristics of Mushroom Cultivation in Plastic Bottles and Small Bags

15.2.1 Plastic Bottles

Most large-scale mushroom production companies, as well as the big cooperative farms in Japan, China, Korea, and Thailand, adopt bottle cultivation. These organizations produce primarily *F. velutipes*, *H. marmoreus*, and *P. eryngii*. They utilize fully automated systems with specialized equipment.

A key advantage of plastic bottle cultivation is that it makes it possible to produce mushrooms in areas with limited land availability. In locations where land prices are high, such as Japan and the suburbs of China's big cities, intensive production in limited space is required. Shelving is not typically used during a significant duration of the bottle process and this greatly increases the density of cultivation rooms.

The use of rigid bottles makes it easy to mechanize large-scale mushroom production, and so automated cultivation equipment can be employed. Production costs are further reduced by the introduction of automated cropping and packaging machines. This new cultivation method saves significant labor by automating the whole production process.

Mushrooms cultivated in plastic bottles are generally produced in controlled-environment facilities, where temperature, humidity, concentration of carbon dioxide, and light illumination are maintained and monitored. Mushrooms can be easily grown year-round in these controlled facilities. In bottle cultivation, mushrooms are usually allowed only one flush, under short cycle times. A great deal of precision is required. Excellent cultivation technology and strict hygiene practices are prerequisites to providing high-yield and high-quality fruit bodies in bottle cultivation. Automation and environmental control contribute greatly to the stabilization and consistency of this intensive production model.

On the down side, these automated facilities require high capital construction inputs and the cultivation machines are not cheap. The initial set-up costs may be too high for many. Energy costs are high due to machinery power demands and environmental control needs.

15.2.2 Small Bags

The materials and supplements used in small plastic bag production are primarily sawdust and agricultural byproducts or residues, such as corncob meal, cottonseed hulls, rice bran, wheat bran, and corn bran. These are generally cheap and easily available throughout the world. Typically, the number of supplements for small bag cultivation is lower in comparison to bottle cultivation. As a result, substrate digestion and colonization are prolonged. However, the total yield of mushrooms is high (bags are often repeatedly flushed), and the biological efficiency is relatively higher than bottles.

Small-scale plastic bag cultivation does not require a big initial investment in large facilities and machinery. All processes can be performed manually in simple structures, for instance, plastic greenhouses (Figure 15.1). Bag cultivation is usually seasonal and is the world's most popular method for the cultivation of specialty mushrooms. In cultivation of *G. frondosa* in Japan, two large-scale production companies produce this fungus in small bags in huge facilities equipped with fully automated machinery (Figure 15.2).

15.2.3 Cultivation Containers

In bottle cultivation, heat-resistant polypropylene bottles with a capacity of 450–1100 ml and mouth openings of 52–78 mm are usually used. Each bottle is fitted with a cap equipped with

Figure 15.1 Small-scale bag cultivation of *Auricularia* in Vietnam.

Figure 15.2 Large facility for bag cultivation of *Grifola frondosa* in Japan.

a microporous-filter disk or polyurethane disk. Since mites invade caps and occasionally live in polyurethane filters, caps without such filters are often used. Small (450–550 ml), medium (600–800 ml), and large-sized bottles (850 to 1100 ml) are used. Figure 15.3 shows a tray containing bottles and several sizes of polypropylene bottles.

Figure 15.3 Polypropylene bottles and tray for cultivation.

In small bag cultivation, the growers generally use plastic bags filled with 1.0–3.0 kg (wet weight) of the mixed substrate. Large-scale mushroom companies use microporous-filtered polypropylene bags and the small-scale growers in Southeast Asia generally use polyethylene bags without a microporous filter that cannot withstand the high temperatures that polypropylene bags can (Figure 15.4).

15.2.4 Substrate Materials

Sawdust, cottonseed hulls, ground corncobs, and straw are the most popular basal ingredients in mushroom cultivation using plastic bottles and bags. A common substrate for specialty mushrooms is composed of the basal ingredients combined with starch-based supplements, such as rice bran, wheat bran, corn bran, millet, maize, and sorghum meal. The mushroom growers of Japan, China, and Korea sometimes use soybean skin, soybean meal, *tofu* refuse, malt feed, sugar cane bagasse, and beet pulp.

15.2.5 Mushroom Cultivation Processes in Plastic Bottles and Small Bags

The common cultivation processes of mushrooms in plastic bottles and small bags are as follows.

1) Preparation of substrate.
2) Mixing of substrate materials.
3) Filling into bottles or bags.
4) Sterilization.
5) Cooling the substrate.
6) Inoculation.
7) Spawn run (incubation).
8) Scratching.

Figure 15.4 Polypropylene small bags with and without microporous filter.

9) Sprouting.
10) Growing.
11) Harvesting.
12) Packaging and shipment.

The mixing, filling, sterilization, and inoculation in small-scale cultivation using plastic bags are often entirely performed by hand. In contrast, the main processes in bottle cultivation are performed by automatic cultivation equipment irrespective of production scale. It is estimated that 92% of worldwide specialty mushroom production is cultivated in small-scale seasonal farms using small bags.

15.2.5.1 Substrate Mixing and Filling

Sawdust or cottonseed hulls are mixed with nutritional supplements and water in a mixer in bottle cultivation (Figure 15.5). The moisture content of the substrate is adjusted to 62–68%, depending on the mushroom species. Extended mixing times occasionally result in the rapid propagation of bacteria, especially during warmer seasons.

The moistened substrate is loaded into the bottle filler. Through the filler, bottles are loaded with a preset quantity of substrate. Once bottles are filled, compactors press the substrate in the bottle down to a preset height and then steel rods are used to put inoculation holes into the compact substrate (Figure 15.6). One to five inoculation holes are generally made. Most bottle growers and production companies also use machines to put on and remove the filter caps.

In small-scale bag culture in Asian countries, the mixed substrate is filled into heat-resistant polyethylene bags by manual labor or by a very simple semi-automatic filling machine (Figure 15.7). Large farms or production companies fill the substrate into bags with automatic filling machines. In the large-scale production companies of *G. frondosa* in Japan,

Figure 15.5 Mixing substrate.

Figure 15.6 Automatic filling machine for bottle cultivation.

microporous-filter patch bags are machine fabricated just prior to automatically filling the substrate into the bags. After filling, 1–5 inoculation holes are punched into the firm cylindrical or cubic substrate block.

Figure 15.7 Filling the substrate for small-scale bag cultivation of Shiitake in China.

15.2.5.2 Sterilization

The filled bottles and bags are loaded into a large autoclave (Figure 15.8). Low-tech bag growers will run sterilization cycles of 5–6 hours at 99–100°C without pressure. Bottle systems commonly use a shorter, hotter, pressurized sterilization cycle of 50–60 min at 118–120°C.

Figure 15.8 Large autoclaves for sterilization of substrate. (*See color plate section for the color representation of this figure.)*

Industrial large-scale pressure vessels are designed with double doors: one for entry and the other for exit of sterile materials.

15.2.5.3 Cooling and Inoculation

The sterilized bottles and bags are cooled to 20–25°C in a cooling room. Slow cooling in a precooling room is advisable because condensation inside the caps occurs when hot bottles are rapidly exposed to cool air. During cooling, the room air flows into the bottles. When the room air is contaminated by microorganisms, the contaminants invade bottles and bags, where they can later propagate. Therefore, the cooling room should utilize strict hygiene and air supplied via HEPA filtration. Cooled bottles and bags are generally transferred from the cooling room through a pass box into the clean inoculation room by a wheel conveyer.

The bottles and the bags are usually inoculated with sawdust spawn using simple or automatic inoculation machines. Some small-scale bag cultivators in Asia inoculate inside low cost wooden boxes in the outdoors. Most mushroom growers using bottles and large-scale companies using small bags usually inoculate using fully automatic inoculation machines in clean rooms (Figure 15.9). The latest automatic machine can inoculate around 7,000–8,000 bottles in 1 h (in a 25 bottle system). Since bottles and bags are opened to introduce the spawn, inoculation rooms must employ high-sanitation HEPA filter systems.

The spawn used by both bottle and bag companies is generally supplied by specialized spawn makers. Large-scale mushroom companies and cooperatives develop hybrid cultivars themselves and make spawn for their own use. Although sawdust spawn is common, recently liquid spawn has gained popularity for large-scale cultivation of *F. velutipes* and *P. eryngii* in Japan, China, and Korea.

Figure 15.9 Automatic inoculation machine for bottle cultivation.

Figure 15.10 Stacking trays containing 25 bottles in incubation room.

15.2.5.4 Spawn Run (Incubation)

In bottle cultivation, 16, 25, and 36 bottles are held in one tray and trays are stacked 8–10 high on a pallet using an automatic palletizer. These pallets are placed on the floor in an air-conditioned incubation room (Figure 15.10). Temperature and humidity in the incubation room is generally maintained between 14–24°C and 65–75%, depending on the mushroom species. The upper limit of carbon dioxide concentration in the incubation room ranges from 2000–4000 ppm. The spawn run (including mycelial maturation) usually takes 21–80 days.

Some of the small, seasonal bag growers incubate in primitive greenhouses or brick houses under natural conditions. They will often organize the inoculated bags directly on the floor of the greenhouse or sometimes place them on shelves in a room. In most year-round Japanese bag cultivation companies, the growers incubate by placing bags on shelves in special environmentally controlled incubation rooms. To save handling, 4–6 substrate blocks are placed in trays, similar to the method of handling bottles.

15.2.5.5 Scratching (Kinkaki) in Bottle Cultivation

Scratching is the mechanical removal of the original inoculum and some surface of the colonized substrate for uniform fruiting in bottle cultivation of *F. velutipes*, *H. marmoreus*, *P. eryngii*, *P. nameko*, and *P. ostreatus*. Automatic scratching machines are equipped with a cap cleaning apparatus for brushing the inside of the caps (Figure 15.11). The scratched surface is sprayed with water to stimulate formation of fruit body primordia and to supply water for sprouting (Medasi).

15.2.5.6 Growing (Seiiku)

The induction of primordia requires low temperature, light, and a low concentration of carbon dioxide. When the scratched bottles are transferred into an induction room that has a lower

Figure 15.11 Scratching machine for fruiting.

temperature and lower concentration of carbon dioxide than the spawn run room, the mycelia shift from vegetative growth to reproductive growth. The environment for pinning and maturation depends on the mushroom species. At the pinning stage, the temperature of the fruiting room ranges from 13–20°C and the humidity from 75–95%. During the growth of the fruit bodies, the colonized substrate is generally maintained at 6–18°C, 85–98% humidity, 1500–2500 ppm carbon dioxide concentration, and 100–1000 lux illumination. For illumination, fluorescent lamps have been traditionally used, but LED technology is spreading.

15.2.5.7 Harvesting and Packaging

While the mushrooms cultivated in small bags can be harvested for approximately 3–5 months by repeated flushing, only one flush of mushrooms is usual in bottle cultivation. Harvested mushrooms are weighed and packaged into plastic bags or containers. In some Japanese large-scale production farms of *F. velutipes*, *H. marmoreus*, and *P. eryngii*, automated harvesting, weighing, and packaging machines are used to save labor costs, the highest of the production expenses (Figure 15.12).

15.2.5.8 Emptying the Substrate

After harvest, the spent substrate is removed from the bottles using automatic emptying machines. These emptying machines operate either by rotary blades or compressed air pressure. Recently, the compressed air emptying machine has become popular in Japan and China. The emptied bottles are washed and reused, while the used bags are thrown away or sometimes washed and recycled. The substrate is usually reused as compost for agriculture. For several years, the spent substrate of *F. velutipes* or *P. eryngii* has been recycled as substrate.

Figure 15.12 Automatic harvesting machine in *H. marmoreus* cultivation.

15.3 Cultivation Methods

15.3.1 *Flammulina velutipes* (Enokitake)

The commercial cultivation of *F. velutipes* began in Japan using 700 ml bottles with an opening of 52 mm in diameter. Later, 850 ml bottles with an opening of 58 to 65 mm became popular. More recently, 1100 ml bottles with an opening of 75–78 mm (16 bottles per tray) and 600–700 ml bottles with an opening of 65–70 mm (25 bottles per tray) are now generally used. While small-scale growers and most large-scale production farms in Japan, China, and Korea cultivate *F. velutipes* in plastic bottles using automated machinery, seasonal small-scale growers in China typically use plastic bags.

In China, large *F. velutipes* farms in the Shanghai area imported Japanese and Korean cultivation machinery at the end of the 1990s. *F. velutipes* production in China has dramatically increased more than 18-fold over the last 16 years from about 150,000 tons in 1997 (Chang and Miles, 2004) to 2,730,000 tons in 2013 (CEFA, 2015). The largest single company produces 160,000 tons annually. In Japan, there are no large companies cultivating *F. velutipes*, and the production by Nagano's cooperative farms is the largest. The annual production in Japan and Korea is 133,000 tons (Japanese Forestry Agency, 2015) and 33,000 tons in 2013, respectively. Nearly all of the 2,930,000 tons global production is cultivated in Asia and so *F. velutipes* is certainly an Asian mushroom.

Up to the mid-1980s, *F. velutipes* growers had to use brown-colored cultivars that turned light brown or brown when exposed to light during growth. However, the market demanded "whiteness" in fruit bodies. Therefore, growers had to cultivate the mushrooms in the dark to

prevent the coloration of caps and stems. Eventually, a novel white strain was developed in 1985. At present, most *F. velutipes* growers use white strains (Yamanaka, 1997).

15.3.1.1 Substrate and Filling

The substrate for *F. velutipes* production was traditionally based on the sawdust of Japanese cedar (sugi, *Cryptomeria japonica*). Most Chinese growers of *F. velutipes* use cottonseed hulls and ground corncobs rather than sawdust. Sawdust, corncob meal, and cottonseed hulls are used as the base ingredients and mixed with nutritional supplements. The recommended formula of substrate for *F. velutipes* cultivation in Nagano is corncob meal (35%: ratio in total dry weight of supplements), cottonseed hulls (5%), rice bran (33%), wheat bran (5%), sorghum meal (5%), dried *tofu* refuse (3%), beat pulp (10%), shell powder (4%), and sugi sawdust. The moisture content of the substrate is adjusted to 65–66%. The wet weight of the substrate filled into the bottles is 68–70 g per 100 ml of bottle capacity. For example, in 700 ml bottles, 470–500 g of the mixed substrate is mechanically filled into the bottle. After filling, 1–5 inoculation holes are punched vertically into the substrate. In small bag cultivation of *F. velutipes* in China, 1–2 kg cylindrical substrate blocks are in general use.

15.3.1.2 Inoculation and Spawn Run

Generally, growers purchase inoculum from spawn manufacturers with the exception of the large-scale company and cooperative farms that often produce spawn themselves. The growers within these cooperatives purchase fully myceliated bottles of liquid spawn grown in the incubation centers of the cooperative. Fifteen to twenty ml of liquid spawn is aliquoted to each bottle.

In bottle cultivation, after sterilization and cooling of the substrate, spawn is inoculated on the substrate surface by automatic inoculation machines. Approximately 9–11 g of sawdust spawn is used for 700–850 ml bottles and these are usually inoculated in strictly clean inoculation rooms. One 800 ml bottle of spawn can inoculate 45–55 bottles.

The inoculated bottles are transferred to a conditioned incubation room kept at 14–16°C, 65–75% humidity, and less than 3000 ppm of carbon dioxide. The spawn run is 21–26 days. Defective sprouting occasionally occurs in *F. velutipes* when the temperature of the substrate exceeds 21–22°C.

15.3.1.3 Scratching and Sprouting (Medashi)

After incubation, both the inoculum and the surface of the colonized substrate are removed by a scratching machine for uniformity of sprouting and fruiting. The scratched surface is also sprayed with water. Scratching should be performed before full mycelial colonization in the cultivation of *F. velutipes*. Waiting until after this time results in reduced number of fruit bodies and yield.

The scratched bottles are placed in sprouting rooms controlled at 13–15°C, 93–95% humidity, and about 1000 ppm of carbon dioxide concentration for 8–12 days. At 6–8 days after scratching, the primordia begin to form. When the young fruit body pilei grow to 1 mm in diameter and the stipe length is 3–5 mm 2–3 days later, the bottles are transferred to the acclimation room.

15.3.1.4 Preceding Period of Growth

15.3.1.4.1 Acclimation (Narashi)

The young fruit bodies dry and abort if, after growing at 13–15°C in the sprouting room, they are abruptly placed into cold conditions of 3–5°C. To counter this, bottles are placed in an

acclimation room maintained at 7–8°C and 90% humidity for 3–4 days to acclimate the fruit bodies in a step-wise manner to the target temperature of 3–5°C, known as restriction.

15.3.1.4.2 Restriction (Yokusei)

After acclimation of the young fruit bodies, the bottles are placed in the restriction room controlled at 3–5°C, 85–90% humidity, and 1000 ppm of carbon dioxide concentration for 5–7 days to restrict the elongation and to equalize the stipe length. Air circulation and illumination are also used to restrict irregular elongation of the stipe.

15.3.1.4.3 Applying the Plastic Collar

When the stipe has elongated about 1–2 cm above the mouth of the bottle, a permeable plastic collar is placed around the bottle neck and secured with a Velcro strip. The collared bottles are moved into the growing room. This collar serves to hold the mushrooms in place so that the development of the pilei is inhibited and the elongation of the stipes is promoted as a result of increased carbon dioxide concentration (Figure 15.13).

15.3.1.5 Late Period of Growth

The growing rooms are maintained 5–7°C, 75–80% in humidity, and 1000 ppm of carbon dioxide concentration. 150–300 lux of illumination for 30–60 minutes per day is required when the stipes reach 6–7 cm long. Excessive humidification in the growing room causes watery mushrooms.

15.3.1.6 Harvesting and Packing

About 25–30 days after scratching, the mushrooms are harvested when the stipes are 13–14 cm (Figure 15.14). Mushrooms are usually vacuum packed in polypropylene bags. *F. velutipes* is traditionally packaged and marketed in a 100-g pack, but now, entire bunches of this fungus are vacuum packaged and shipped. The average yield in bottle cultivation is in the range of

Figure 15.13 Fruit body development of *F. velutipes* after scratching.

Figure 15.14 *F. velutipes* fruiting in bottle cultivation. (*See color plate section for the color representation of this figure.)*

210–240 g per 700 ml bottle containing 480–520 g of substrate and 300–340 g per 1100 ml bottle containing 750–810 g of substrate. Biological efficiencies are 127–145% and 100–130%, respectively. In small bag cultivation of this fungus in China, the total yield in three croppings is around 400 g in 1.0 kg of substrate (Figure 15.15).

Figure 15.15 *F. velutipes* fruiting in small bag cultivation in China.

15.3.2 *Hypsizygus marmoreus* (Buna-shimeji)

The cultivation of *H. marmoreus*, which originated in Nagano, Japan, is now widespread throughout Japan and China. Large-scale mechanized facilities with production capacities of 10 tons per day are found throughout both nations. The present annual production of this fungus is 117,000 tons in Japan and 146,000 tons in China.

15.3.2.1 Substrate and Filling

Commercial production of *H. marmoreus* is commonly on a substrate of sawdust or corncobs contained in 850 ml bottles with an opening between 58–65 mm in diameter. Recently, growers and large-scale production companies use smaller 450–700 ml bottles with an opening of around 60 mm. The inside of the cap typically has a projecting structure that presses the spawn surface lightly

The substrate for *H. marmoreus* is based on pine wood sawdust and corncobs. In Nagano, a substrate consisting of 20 g (wet weight) of corncobs, 50 g of rice bran, 20 g of wheat bran, 20 g of soybean hull, 10 g of cottonseed hulls, 10 g of dry *tofu* refuse and sawdust in 850 ml bottles is recommended. Around 450 g of the mixed substrate in 700 ml bottles and around 510 g of the substrate in 850 ml are mechanically filled into the bottle. After filling, one inoculation hole is generally punched into the substrate, simultaneously compacting the substrate within the bottle. Some growers punch 3–4 inoculation holes into the substrate to shorten the period for spawn run and mycelial maturation.

15.3.2.2 Inoculation and Spawn Run

Generally, growers of *H. marmoreus* purchase inoculum from spawn manufacturers. Large-scale companies and cooperatives develop hybrid strains and use spawn made in-house. Some growers belonging to farmers' cooperatives purchase pre-inoculated and colonized substrate from the cooperative's incubation centers. All they need do is fruit the bottles for cropping and packing.

After sterilization and cooling the substrate, the spawn is inoculated on the substrate surface with automatic inoculation machines. The amount of spawn for *H. marmoreus* is greater than for other species, because fruiting will take place directly from the surface of that spawn. The usual dose is between 13–15 g of sawdust spawn for a 850-ml bottle and 12–13 g for a 700-ml bottle. Primarily gray-brown strains of *H. marmoreus* are cultivated in Japan, however, one large production company cultivates a white strain too.

The inoculated bottles are placed in an environmentally controlled incubation room conditioned at 21–23°C, 65–75% humidity, and less than 4000 ppm carbon dioxide concentration. After a 28–35-day spawn run, the bottles are incubated for an additional 32–45 days of mycelial maturation. Trays containing 16, 25, or 36 bottles are piled up on a pallet using automatic palletizers to a height of about 8–10 trays (Figure 15.10). When using trays that accommodate 25 or 36 bottles, 1–4 bottles are pulled out of the center of the tray to avoid an undesirable substrate temperature rise.

15.3.2.3 Scratching and Growing

Specialized scratching is performed in the cultivation of *H. marmoreus*. After spawn run and complete mycelial maturation, only the peripheral portion of the spawn that covers the substrate surface is removed (Figure 15.16). This leaves the central area of the spawn untouched. Fruiting will occur directly from this area. The scratched surface is lightly sprayed with water.

Scratched and sprayed bottles are covered with perforated plastic sheeting and are moved into a growing room conditioned at 14.5–16.0°C, 96–98% humidity, around 2000 ppm of carbon dioxide concentration, and 50–100 lux of illumination. Nine to ten days

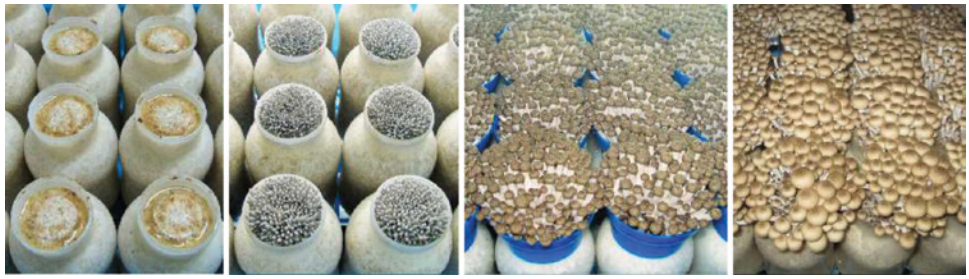

Figure 15.16 Fruit body development of *H. marmoreus* after scratching. (*See color plate section for the color representation of this figure.)*

after scratching, when the young fruit bodies grow and contact the plastic sheeting, the covering is removed. Then the bottles are kept in an environmentally controlled room at 14.5–16°C, 96–98% humidity, 1500–1800 ppm carbon dioxide, and 800–1000 lux (15–30 min for 12 h per day). Mushrooms are harvested 21–24 days after scratching, depending on strain (Figure 15.17).

15.3.2.4 Harvesting and Packaging

The average yield is in the range of 190–220 g per 700 ml bottle containing 450 g of substrate and the biological efficiency is 120–140%. This fungus has been traditionally packaged in 100 g polystyrene containers by splitting the bunch. In recent years, packaging an entire bunch into plastic bags without splitting has become common. An excellent cultivation technique is required to employ bunch packing because predictably equal bunch weights must be consistently produced.

Figure 15.17 *H. marmoreus* fruiting in bottle cultivation.

15.3.3 *Pleurotus eryngii* (King Oyster)

Bag cultivation of *P. eryngii* by burying the myceliated colonized blocks underground began in the late 1970s or early 1980s in northern Italy (Ferri, 1985). Bottle cultivation of this fungus began in Taiwan around the end of the 1980s. Thereafter, production has increased rapidly in recent years after a large-scale Japanese production company began experimental bottle cultivation in 1992. At present, bottle cultivation of *P. eryngii* has spread in China, Korea, and Thailand because this fungus is delicious and possesses a long shelf life compared to the other *Pleurotus* species. The production of *P. eryngii* in 2013 was about 40,000 tons by bottle cultivation in Japan and 673,000 tons in China, which includes production both in bottles and in bags.

15.3.3.1 Substrate and Filling

Most growers of *P. eryngii* have generally used a substrate of sawdust, corncob meal, and cottonseed hulls contained in 850 ml bottles. The large-scale production companies use small 450–700 ml bottles with an opening of 52–64 mm.

The substrate for *P. eryngii* production is based on the sawdust of Japanese cedar and corncobs. To the mixed sawdust and corncobs are added 70–80 g (wet weight) of supplement consisting of rice bran, wheat bran, and dried *tofu* refuse. The mixed substrate is adjusted to 65–68% moisture content. The 850 ml bottles contain 510–530 g of wet substrate. In a substrate using only corncobs as the base material, 130 g (wet weight) of corncobs, 20 g of rice bran, 20 g of wheat bran, 20 g of sorghum meal, 30 g of soybean skins, and 15 g of dried *tofu* refuse are mixed and 550–580 g of the substrate fills a 850 ml bottle.

15.3.3.2 Inoculation and Spawn Run

The growers of *P. eryngii* purchase inoculum from spawn manufacturers. Large-scale production companies and large cooperatives develop new hybrid strains for their own use. The 850 ml bottles are usually inoculated with 10–13 g of sawdust spawn using automatic inoculation machines in a clean room. Liquid spawn is usually used in the production of *P. eryngii* in China and Korea. Fifteen to twenty ml of liquid spawn is inoculated in this case.

The inoculated bottles are placed in an environmentally controlled incubation room conditioned at 21–23°C, 65–80% humidity, and less than 4000 ppm of carbon dioxide. After a 23–25-day spawn run, the bottles are incubated for an additional 4–6 days. The environmental management is very important because the colonizing mycelia of *P. eryngii* differentiate easily into fruit body primordia on top and within the bottle under low temperature air flow and bright illumination.

15.3.3.3 Scratching and Growing

After incubation, the spawn and the surface of the colonized substrate are removed. The scratched surface is sprayed with a little water. Then bottles are covered with perforated plastic sheeting or old newspapers and transferred into a growing room conditioned at 14–16°C, 75–95% humidity, less than 3000 ppm of carbon dioxide concentration, and 100–200 lux of illumination. Another method for sprouting is flipping the bottles upside down. The sprouting period required for this flipping method is prolonged compared with the standard method.

When the young fruit bodies grow and make contact with the plastic sheet or newspaper, the covering is removed. Then the bottles are placed in an environmentally controlled room at 14–16°C, 75–95% humidity, less than 3000 ppm carbon dioxide, and 100–500 lux illumination.

Mushrooms are harvested 13–16 days after scratching. Recently, growers began using LED illumination systems instead of fluorescent lighting (Figure 15.18).

Figure 15.18 *P. eryngii* fruiting in bottle cultivation under LED illumination. (*See color plate section for the color representation of this figure.)*

15.3.3.4 Harvesting and Packaging

The yield of *P. eryngii* is in the range of 160–190 g per 850 ml bottle and 150–180 g per 700 ml bottle. The biological efficiencies are 70–100% and 90–110%, respectively. After trimming the stems near the base with a knife, the fruit bodies are weighed and packaged into plastic containers or plastic bags (Figure 15.19).

Figure 15.19 Packaging of *P. eryngii*.

15.3.4 *Pholiota nameko* (Nameko)

P. nameko is originally from the highlands and high latitudes of Japan and has traditionally been eaten by the people of northern Japan. So, the production of *P. nameko* began with log cultivation around 1921 in the Tohoku district (northeastern Japan). In the 1960s, wooden tray cultivation and small bag cultivation using sterilized sawdust substrate spread in eastern Japan. Bottle cultivation was developed in the early-1980s and at present, this production method is the most popular in Japan. The production of *P. nameko* in 2013 was about 23,000 tons in Japan and 960,000 tons in China. The production of *P. nameko* is rapidly gaining popularity in other Asian countries.

15.3.4.1 Substrate and Filling

The growers of *P. nameko* generally use a substrate of hardwood sawdust using 800 ml bottles with 78 mm openings. Supplements such as corn bran, wheat bran, and dried *tofu* refuse in the ratio 10% of total fresh substrate weight are mixed with sawdust. The mixed substrate is adjusted to a 64–65% moisture content and bottles contain 540–560 g of wet substrate.

15.3.4.2 Inoculation and Spawn Run

The growers of *P. nameko* generally purchase 850–1500 ml spawn bottles from spawn manufacturers. One 1500 ml sawdust spawn bottle can inoculate 100–150 bottles. The inoculated bottles are placed in an incubation room maintained at 16–18°C in the early period (22–23°C in the latter half of the spawn run), 65–80% humidity, and less than 2500 ppm of carbon dioxide. Spawn run of *P. nameko* usually takes 45–65 days, depending on the strain.

15.3.4.3 Growing and Harvesting

The spawn and the substrate surface are mechanically removed with a scratching machine and the scratched surface is sprayed with water. Afterwards, the bottles are placed upside down in a growing room at 14–16°C, more than 90% humidity, less than 2000 ppm of carbon dioxide, and around 200 lux illumination in daytime. Seven to ten days after scratching, primordia begin to form. 25–40 days later, the mushrooms are harvested (Figure 15.20). Average yield is 180–200 g per 800 ml bottle. The biological efficiency is 95–115%.

15.3.5 *Pleurotus ostreatus* (Oyster Mushroom, Hiratake)

It is estimated that 2013 global production of *Pleurotus* spp. (*P. eryngii* excluded) reached 6,340,000 tons including 6,150,000 tons produced in China. Most of the production is cultivated using small bags. *P. ostreatus* production in Japan peaked in 1989 at 35,700 tons (Yamanaka, 1997) and the production was only 2,290 tons in 2013 (Japanese Forestry Agency, 2015), a decrease of more than 16-fold in 24 years. The reason is because *P. ostreatus* growers shifted to *P. eryngii* production. Ninety-nine percent of the total production is by small or big bag cultivation using sterilized or non-sterilized substrate by small-scale growers. Most small-scale *P. ostreatus* growers in Japan and some growers in Korea produce using bottle cultivation.

15.3.5.1 Substrate and Filling

Conifer sawdust is used as the base medium for bottle cultivation in Japan and 850 ml bottles with 58–65 mm openings are generally utilized. Supplements such as rice bran, wheat bran, and hominy feed are mixed with sawdust and the substrate is adjusted to 64–67% moisture content; 480–500 g of substrate is filled into plastic bottles and the bottles are sterilized in an autoclave.

Figure 15.20 *P. nameko* fruiting in bottle cultivation. (*See color plate section for the color representation of this figure.*)

The small bag growers of this fungus in China and other Asia countries mostly use cottonseed hulls, wheat or rice straw, corncobs, rice bran, wheat bran, and sugarcane bagasse for the ingredients. The substrate is pasteurized or is nearly sterilized at 98–100°C in a steaming chamber at atmospheric pressure. Bags are typically 1–3 kg wet weight.

15.3.5.2 Inoculation and Spawn Run

In bottle cultivation, an 850-ml sawdust spawn bottle will inoculate about 40–50 bottles. The inoculated bottles are placed in an incubation room maintained at 21–23°C, 65–75% humidity, and less than 4000 ppm of carbon dioxide for 22–25 days. After the spawn run, the bottles are incubated an additional 3–5 days. In small bag cultivation, 20–30 g of spawn is inoculated to the bag and the inoculated bags are incubated at 23–27°C for 35–50 days.

15.3.5.3 Growing and Harvesting

The colonized substrate of *P. ostreatus* in bottle cultivation is usually scratched. After the scratching and water spraying, bottles are placed in a growing room at 14–16°C, more than 90% humidity, less than 2000 ppm of carbon dioxide and 200–500 lux illumination. The bottles are put upside down for sprouting. Five to seven days after scratching, the fruit body primordia form. Around 1 week later, the mushrooms are harvested (Figure 15.21). The yield is 100–120 g per 850 ml bottle. The biological efficiency is 60–80%, which is the lowest of the cultivated mushrooms. In the case of small bag culture, 3–4 flushes are possible. Therefore, the total yield of mushrooms is 800–900 g per 2.5 kg block in bag cultivation and the biological efficiency ranges from 90 to 110%.

15.3.6 *Grifola frondosa* (Maitake)

The production of *G. frondosa* began with sawdust cultivation using plastic bags in the Tohoku district of Japan around 1975. Large-scale production of this mushroom by bag cultivation started in 1983, and the production rapidly increased in eastern Japan. The volume sold in 2013

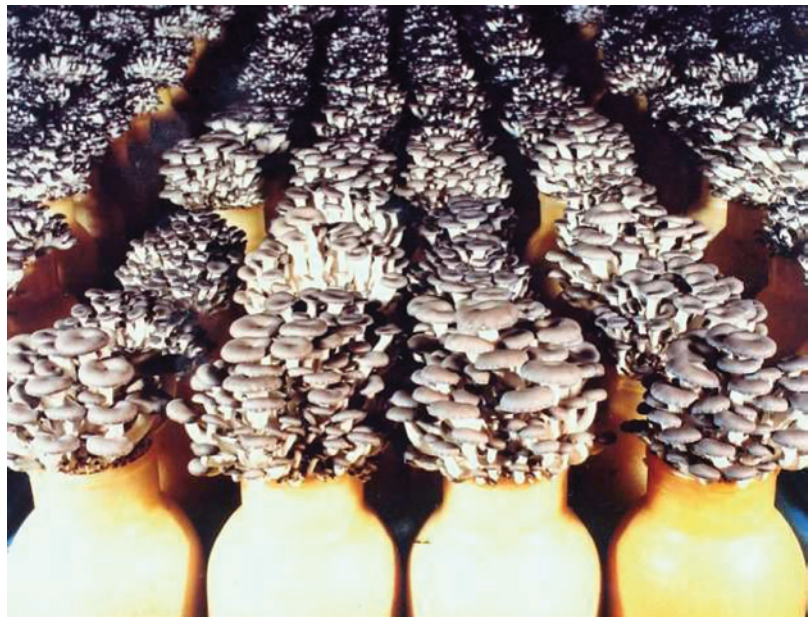

Figure 15.21 *P. ostreatus* fruiting in bottle cultivation.

was 45,450 tons in Japan and 21,000 tons in China. The majority of worldwide production takes place in Japan and China. In Japan, 76% of the production is cultivated in polypropylene small bags by general growers and two large-scale companies. The remaining 24% is cultivated in plastic bottles by a large mushroom production company. Amazingly, 84% of *G. frondosa* production in Japan is cultivated by only three mushroom companies (Yamanaka, 2011).

15.3.6.1 Substrate and Filling

A substrate base of hardwood sawdust and hardwood chips is generally used in bottle and bag cultivation. Companies employing bottle cultivation use bottles of around 750 ml in volume. Supplements such as rice bran, wheat bran, corn bran, and dry *tofu* refuse are mixed with sawdust, and the substrate is adjusted to a 63–66% moisture content.

Small bag cultivators of this fungus in Japan and China mostly use sawdust, wood chips, cottonseed hulls, rice bran, and wheat bran for the ingredients. They usually fill bags with 1–3 kg of substrate. In Japanese bag cultivation, 80% of the production is cultivated in 2.5–3.0 kg blocks and 20% is cultivated in 1.5–1.7 kg blocks. Growers usually use polypropylene bags with heat-sealed permeable filters (Figure 15.4). The moistened substrate is filled into bags with semi-automatic or automatic filling machines, compacted, and reamed with 4–6 inoculation holes.

15.3.6.2 Inoculation and Spawn Run

The typical growers of *G. frondosa* purchase inoculum from spawn manufacturers, however three large-scale companies in Japan have developed new hybrid strains and operate their own spawn divisions. Though commercial strains are popular in gray-black, the two large-scale bag companies also cultivate white isolates. Ten to fourteen grams of spawn is inoculated to a 2.5 kg substrate block.

The inoculated bags are placed in the incubation room maintained at 20–23°C, 65–70% humidity, and less than 2000 ppm of carbon dioxide for 30–50 days, depending on strain, substrate, and cultivation method. Thereafter, a white mycelial mass (primordia) is formed and swells on the surface of the substrate.

15.3.6.3 Growing and Harvesting

When the mycelial mass changes color to a dark-gray or black in the incubation room, the bags are transferred into a sprouting room maintained at 20°C, 75–90% humidity, and 250–600 lux illumination. Upon moving into the sprouting room, the bags are cut to allow the primordial mass to develop. In another sprouting method, the bags are cut after the mycelial blocks are transferred into a growing room controlled at 17–19°C, 92–95% humidity, less than 1000 ppm CO_2, and 600–1,000 lux illumination.

Four to five days after cutting the bags, black and large primordia rapidly develop and differentiate into young fruit bodies by exposure to fresh air. Blue light is used to make the pilei darker during the latter half of development because Japanese consumers prefer dark-black pilei over gray. The fruit bodies are harvested 8–10 days after cutting the bags (Figure 15.22). In Japanese cultivation of *G. frondosa*, only one crop is usual and the yield from a 2.5 kg substrate block is 480–650 g (biological efficiency is 56–76%) and the yield from 1.1 kg of substrate is in the range of 220–300 g (biological efficiency is 60–80%). The best yield of 400–500 g (biological efficiency 74–92%) is obtained from 1.6 kg mycelial blocks cultivated using an excellent hybrid strain and the best substrate. The cluster of fruit bodies of *G. frondosa* are usually sliced, weighed, and packaged.

15.3.7 *Lentinula edodes* (Shiitake)

Dried Shiitake production by log cultivation in Japan has decreased gradually because it requires hard labor, growers are aging, and there is a deficiency of high-quality logs. Additionally, the

Figure 15.22 *G. frondosa* fruiting on colonized substrate in bag cultivation. (*See color plate section for the color representation of this figure.)*

number of logs produced in the Tohoku district decreased sharply because many oak trees for log cultivation suffered from radioactive contamination following a nuclear plant accident in 2011.

In small bag cultivation of *L. edodes*, the cultivation cycle is short compared with log cultivation. Labor costs are saved by taking advantage of mechanization and the use of small and light mycelial blocks. Year-round production is possible by modestly equipped small plants. For these reasons, production of fresh *L. edodes* in bag cultivation has rapidly increased since 1993 in Japan. Bag-grown product accounts for about 89% of fresh Shiitake and 65% of the total Shiitake production (including the dry Shiitake production converted in wet weight).

On the other hand, *L. edodes* has been produced traditionally by small bag cultivation in China. Chinese Shiitake production in 2013 was about 7,100,000 tons and the quantity is estimated to be 98% of worldwide Shiitake production.

15.3.7.1 Substrate and Filling

Two main types of cultivation methods are popular in Japan. The first model has growers purchase bags, sawdust, and supplements, along with spawn from spawn manufacturers. With the technical guidance of the spawn manufacturers, growers control the entire cultivation cycle themselves from substrate through to harvesting. The second model has growers purchasing fully colonized mycelial blocks from the spawn manufacturers and they then cultivate from sprouting through to harvesting. Lately, there has been a considerable increase in Japanese Shiitake growers purchasing fully colonized mycelial blocks from China.

The substrate is based on 30–40% hardwood chips mixed with hardwood sawdust. Supplements such as rice bran, wheat bran, and corn bran are mixed with sawdust, and the substrate is adjusted to a 62–64% moisture content. In China, cottonseed hulls and corncobs are also popular basal ingredients. The nutritional supplements are generally added to the mix at a 10–12% ratio (wet weight) of the substrate. The moistened substrate is poured into bags with 1–2 microporous filters by semi-automatic or automatic filling machines. Four to six inoculation holes are vertically opened after the substrate is pressed into a cubic or cylindrical form. General weight of the substrate is 2.5–3.3 kg. One spawn manufacturer sells 1.1 kg colonized substrate blocks.

15.3.7.2 Inoculation and Spawn Run

Most growers of *L. edodes* purchase their inoculum from spawn makers. After sterilization and cooling, 15–22 g of spawn is inoculated to a 2.5 kg substrate. The inoculated bags are placed in an incubation room maintained at 18–21°C in the early incubation period, thereafter, in the latter half of the incubation period at 20–22°C, 65–70% humidity, and less than 3000 ppm of carbon dioxide. Mycelia colonize the substrate within 28–35 days, depending on strain and substrate formula, and the incubation requires an additional 50–70 days for mycelial maturation. During this time, the substrate surface changes color from white to dark brown and all, or part, of the bags are shifted to a fruiting environment.

15.3.7.3 Primordia Formation

Fruit body primordia begin to form under the surface of the colonized substrate block in an environment of 18–20°C, 60–80% humidity, less than 2000 ppm carbon dioxide concentration, and 100–300 lux illumination. After maintaining this condition for 10–20 days, the colonized substrate blocks are transferred into the growing room.

15.3.7.4 Growing and Harvesting

Although, the optimum temperature for fruit body growth is 15–18°C, growers typically employ a broad temperature fluctuation regimen during which the highest temperature is

22–23°C and the lowest is 12–13°C. Fruit bodies of *L. edodes* develop at 80–85% humidity, less than 2000 ppm and 100–1,000 lux illumination. Ten to twenty days after spraying water over the blocks, the mushrooms are harvested over a 7–20-day period, depending strongly on the strain. After a flush, growers use a rest-dehydration-rehydration cycle to initiate additional flushes – up to five or six over 3–4 months. The yield from a 2.5 kg substrate is 1.0–1.1 kg and the biological efficiency is 105–120%.

Two types of flushing methods for *L. edodes* cultivation have become popular in Japan. One is the traditional flushing from the entire surface of the block after removing the colonized substrate completely from the bag. The other is flushing from only the top surface of the block after cutting or turning down the bag to expose a quarter of the block.

15.3.7.4.1 Entire Surface Flushing Method

Mushrooms are flushed from the top and all side surfaces of the block after humidifying or spraying. The main advantages of this flushing method are early flushing and limiting damage to the colonized blocks. On the other hand, humidifying or spraying requires labor, and cropped mushrooms are sometimes damaged by watering. Occasionally, the colonized blocks are placed at 28°C before removing the bags to harvest fewer, larger, high-quality fruiting bodies (Figure 15.23).

15.3.7.4.2 Top Surface Flushing Method

Water is filled into the bag that has been turned down, and thereafter the mushrooms flush mainly from the top surface of the block. Spraying the blocks and injecting water into them is unnecessary. Therefore, quality degradation by overhydration is avoided. Because growth from the sides and the bottom of the block is prevented, large and high-quality fruit bodies are produced from the top surface. To reduce the number of fruit bodies, the colonized blocks are often placed at 27–28°C before flushing. Recent work has found that further benefit is possible by exposing the top surface 3–4 weeks earlier than usual, spraying with water and keeping at

Figure 15.23 *L. edodes* fruiting on colonized substrate removed from bags (*See color plate section for the color representation of this figure.*)

20–23°C. Such treatment can encourage flushing 2–3 weeks earlier than the usual high temperature regimen. Discolored water in the bag should be regularly drained away, allowing the water-covered surfaces exposure to fresh air prior to refilling the bags with water.

15.3.7.5 Resting

The mycelial blocks are rested in the growing or resting room maintained at 20–25°C, 60–90% humidity, less than 2000 ppm of carbon dioxide concentration, and 50–100 lux for 12–15 days. In the entire surface flushing system, the blocks are rested at 16–22°C after spray washing the microorganism off the block surface at 23–25°C for 2 days. In the entire surface flushing, it is possible to flush continuously for 5–6 months by spraying water onto the blocks for a few hours per day under fluctuating temperature conditions. The resting period in the top surface system is generally 3–7 days under high temperature conditions of 23–27°C.

15.4 Diseases in Mushroom Cultivation in Plastic Bottles and Small Bags

In mushroom cultivation in plastic bottles and small bags, all living microorganisms in the growth substrate are essentially killed (deactivated) by sterilization autoclaving. Therefore, the fungal and bacterial contamination of substrate is generally caused by use of contaminated spawn, airborne spores, contaminated dust, mites, and contaminated workers. Most contamination is the result of:

- Infection by airborne fungal spores and/or bacteria of the substrate during post-sterilization cooling and inoculation.
- The invasion of mites carrying fungal spores and bacteria into bottles during spawn run.
- Bacteria and the spores of imperfect fungi occasionally contaminate the substrate through pin holes at the bottom of bags.

15.4.1 Fungal Diseases

15.4.1.1 *Trichoderma* Diseases

Mycoparasitic *Trichoderma* (green mold) is a ubiquitous soil fungus and is the common mushroom contaminant in cultivation in bottles and bags. *Trichoderma* spp. proliferated into the substrate degrade not only active mushroom mycelium but the mycelium in spawn. *Trichoderma* can completely prevent mushroom fruiting and cause serious damage to crops. *T. viride* and *T. harzianum* are well known as the most serious and damaging contaminants in bottle cultivation of *Hypsizygus marmoreus* and in bag cultivation of *Lentinula edodes*.

Serious *Trichoderma* diseases are frequently found in association with mushroom mites. The mites feed on the mycelium and the conidiospores of *Trichoderma* and *Penicillium* spp. and then carry these into uninfected bottles. Some mite species have pouches on their bodies that hold mold spores and mycelium. This increases the likelihood that they will "seed" their own food as they travel about. The population of mites increases explosively with propagation of *Trichoderma*. The mites move from contaminated bottles to uninfected bottles until finally *Trichoderma* contamination expands throughout the incubation room. An incubation room heavily populated by mites can result in 100% of newly inoculated bottles becoming lost to contaminant infection in less than 7 days. In general, the fruit bodies of specialty mushrooms are hardly affected by *Trichoderma* spp. and do not develop cap spotting. In bag cultivation of *L. edodes*, *Trichoderma* spp. sometimes infect wet block surfaces and the cut-off stumps that remain on blocks after harvesting.

For protection against *Trichoderma* diseases in bottle cultivation, disinfectant mats should be placed at the entrance of cultivation rooms. Bottles and bags contaminated by *Trichoderma* must be removed from incubation rooms immediately upon finding these bottles. The contaminated substrate should be emptied from bottles and bags after a low temperature autoclaving of about 80–100°C.

15.4.1.2 Cobweb Disease, *Cladobotryum* Infection

Several species of *Cladobotryum* spp. are well known, common pathogens causing cobweb disease in *Agaricus* cultivation (Fletcher and Gaze, 2008). Cobweb disease ("cottony mold disease" in Japanese) has been observed in bottle cultivation of *Flammulina velutipes*. Recently this disease has frequently been found in bottle cultivation of *H. marmoreus* and *Pleurotus eryngii*. The species causing cobweb disease in *F. velutipes* and *H. marmoreus* is identified as *C. varium*. The species *C. mycophilum* found on *P. eryngii* produces a yellowish-red pigment (Back et al., 2012). Occasionally, infected fruit bodies of *P. eryngii* turn a pink color.

Contamination of substrate by this mold is rare. The typical symptom of cobweb diseases in bottle and bag cultivation is a fluffy, down-like growth of mycelium over the young fruit bodies of *F. velutipes* (Figure 15.24a) and *H. marmoreus* (Figure 15.25). Affected fruit bodies are gradually enveloped by a soft powdery mycelium (Figure 15.24b). In *P. eryngii*, *Cladobotryum* colonizes mainly by crawling up the stem. Sometimes, mycelium of the pathogen grows on the stem of *P. eryngii* after packaging, while on the market.

Contaminated mushrooms, *Cladobotryum*-infected debris, and airborne spores in growing rooms are the initial infection sources. To avoid the spread of infection, all primary pathogen sources should be removed from the growing room through strict cleaning. *Cladobotryum* spp. prefer to grow in wet, dead-air pockets such as the corners of the growing room. Environmental control such as absolute humidity control and efficient ventilation in the growing room is very important to prevent cobweb disease in bottle cultivation. Vigorous and healthy fruit bodies are able to escape infection with cobweb disease because *Cladobotryum* spp. are opportunistic pathogens that attack weak mushrooms.

In bottle cultivation of *F. velutipes*, reused plastic collars contaminated with *Cladobotryum* can cause the disease. Contaminated plastic wrapping collars must be washed and disinfected before reuse.

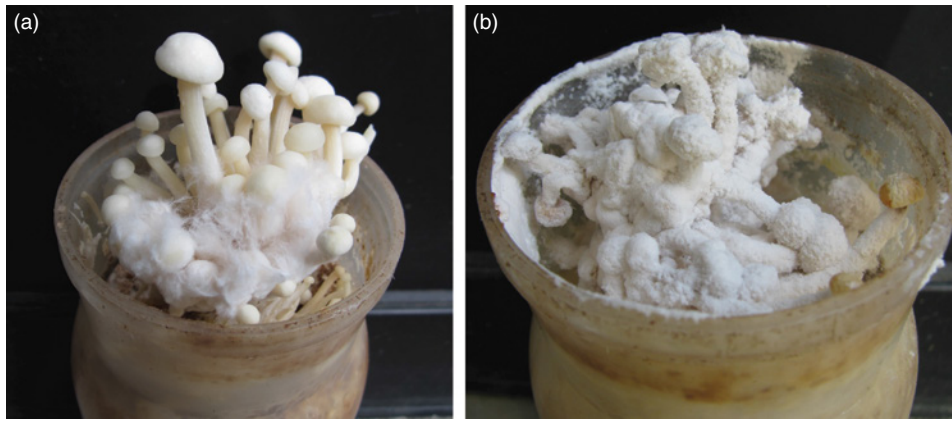

Figure 15.24 Fruit bodies of *Flammulina velutipes* affected by *Cladobotryum varium*. Mushrooms covered with the fluffy mycelium in the early stage (a) and enveloped with soft powdery mycelium at a later stage of disease development (b). (*See color plate section for the color representation of this figure.)*

Figure 15.25 *C. varium* attacking fruit bodies of *Hypsizygus marmoreus*.

15.4.1.3 Other Fungal Diseases

Imperfect fungi such as *Penicillium*, *Aspergillus*, *Cladosporium*, and Zygomycetes such as *Mucor* and *Rhizopus* are extremely common contaminants in bottle and bag cultivation facilities. These contaminants are not mycoparasitic pathogens for mushroom mycelium but are antagonistic molds or weed molds growing in the substrate. The mushroom's mycelial growth is seriously inhibited by fast-growing weed molds as they compete for food in the confined space of a bottle. Consequently, the molds inhibit mushroom fruiting and cause crop losses.

Zygomycetes rapidly grow in bottle and bag substrates. It is fully possible for mushroom mycelium to grow through a substrate that has been previously colonized by one of these contaminants. When this happens, the contamination is difficult to distinguish from mushroom mycelium by the end of spawn run. Occasionally therefore, spawn contaminated with Zygomycetes is used as inoculum and this is unnoticed.

After autoclaving, condensed dew is usually formed on the underside of the caps during substrate cooling. When the cooling room and the incubation room are contaminated by airborne microorganisms, contaminated dew (including mold spores and bacteria) fall down on the substrate surface. Mold and bacteria inoculated onto substrate in this manner frequently form diamond-shaped or mountain-shaped contamination patches in the bottle neck (Figure 15.26).

Neurospora crassa (pink mold) is one of the fastest growing contaminants and spreads numerous airborne spores. The bottles or blocks contaminated with this mold, though sealed, can spread and infect most nearby bottles or bags placed in an incubation room. All affected bottles and bags should be removed as soon as possible from the incubation room. Once the mold propagates in an incubation room, elimination is very difficult. Remnants of substrate and neglected waste substrate under high humidity and warm temperature conditions frequently become the initial cause of *Neurospora* contamination.

Major contamination caused by the previously mentioned competitor molds can be prevented by standard sanitation and strict hygiene of the cooling, inoculation, and incubation rooms.

15.4.2 Bacterial Diseases

In bottle and bag cultivation in Japan, *Bacillus subtilis* is the most common bacterium that severely inhibits the growth of mushroom mycelium. Mycelial growth is strongly prevented

Figure 15.26 Antagonistic contaminants competing with mushroom mycelium. The diamond-shaped patches show that the contaminants were introduced into the bottles during the cooling of substrate or inoculation.

when substrates are precolonized by *B. subtilis* (Figure 15.27). Endospores of *Bacillus* naturally occur in substrate ingredients (air- or soil-borne). These are able to survive at high temperatures and pressures within an autoclave. Infection of the endospores of *B. subtilis* is also caused by contaminated air flow into bottles and bags during substrate cooling, even when the substrate is a high temperature at 100°C. A principal pathogen of bacterial diseases in mushroom cultivation is *B. subtilis* var. *natto* that produces the Japanese traditional food *natto* (a fermented soybean). In Japan, therefore, workers who have eaten *natto* at breakfast are strictly forbidden

Figure 15.27 Bacterial contamination in the substrate (right). Mushroom mycelial growth is strongly prevented by preceding fully colonized bacteria.

to inoculate spawn. This is especially important for spawn making. *Natto* food remnants can stick to the mouth and hands and as they dry, the endospores form. These can easily then become airborne and cause bacterial contamination transfer to the spawn and general inoculation room.

Pseudomonas spp. and other competitor bacteria occasionally contaminate substrate during spawn run. Unnoticed, negligible bacterial contamination in spawn can expand extensively. Most common pathogenic bacteria, such as *Pseudomonas tolaasii*, cause a bacterial blotch or a discoloration of fruiting bodies of *F. velutipes*, *P. ostreatus* cultivated in bottles, and *L. edodes* cultivated in bags. Mushrooms should be grown under a favorable environment, because fruit bodies grown under wet and poor-air-circulation condition are readily affected by bacteria. Strict hygiene throughout the cultivation facility is most important to prevent infection by bacterial and fungal contaminants.

15.5 Pests in Mushroom Cultivation in Plastic Bottles and Small Bags

Mites are the most serious pests of mushrooms cultivated in bottles, especially *H. marmoreus*. *Tyrophagus putrescentiae*, *Pygmephorus mesembrinae*, and *Tarsonemus* sp. are common mushroom mites in bottle cultivation of specialty mushrooms. These mites preferably feed on spores and mycelia of *Trichoderma* and *Penicillium* molds as well as bacterial contaminants. Figure 15.28 shows patch-like blue-green colonies of *Penicillium* caused by an invasion of mites fed on spores. Such patch-like contamination patterns indicate the moving traces of mites in the substrate.

Once female mites invade bottles, the mold colonizes prolifically and the mite population increases exponentially. *Trichoderma* carried by mites completely degrades the mushroom mycelia in the substrate and thereafter the mites in contaminated bottles migrate into adjacent

Figure 15.28 Patch-like blue-green colonies of *Penicillium* caused by an invasion of the mites fed the spores. Patch-like contamination patterns show the moving trace of mites in the substrate.

newly inoculated bottles. If a mite-infested, mold-contaminated bottle is left in the incubation room for a long time, the mite population within the bottle becomes overpopulated. When this occurs, the mites evacuate the bottle all together and invade the incubation room in search of new food – and finding newly inoculated, uncontaminated bottles. At the last stage of serious mite infection associated with *Trichoderma*, the reddish-brown-colored shed skins of mites are frequently clustered on the cap and shoulder surface of bottles and also on the floor.

In bottle cultivation of *H. marmoreus*, it is well known that mites can invade bottles only 1 to 5 days after inoculation, prior to colonization of the substrate surface by mushroom mycelium. Therefore, incubation under a strict, hygienic environment in the early period of spawn run is very important to protect from mite invasion. The thorough removal of dust from the floor of incubation rooms using a vacuum cleaner is the most effective method to decrease the population of mites. Puddles on the floors of incubation rooms accelerate mite propagation because mushroom mites prefer to inhabit dark and wet places, such as floor cracks and underneath pallets.

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16

Cultivation of *Pleurotus ostreatus*

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16.1 *Pleurotus* Species as Edible Fungi

Pleurotus is a genus of the phylum Basidiomycota that is characterized by basidiomata (mushrooms) with oyster-shaped pilei, eccentric stalks (with some exceptions), and the ability to secrete a variety of enzymes to degrade lingo-cellulosic wastes (Rajarathnam and Bano, 1987). There are 50 valid species recognized in this genus, although up to 1000 have been described around the world (Guzman, 2000). Approximately 20 species and varieties of *Pleurotus* are commercially cultivated on a wide variety of substrates using slightly different technologies (Table 16.1, Figure 16.1.a–c) (Buchanan, 1993). Thus, this genus is rendered as one of the most diverse groups of edible and domesticated mushrooms (Vilgalys et al., 1996). *Pleurotus ostreatus,* commonly known as the oyster mushroom, is most commonly cultivated and consumed in south East Asia, India, Africa, and Europe (Mandeel et al., 2005). This species possesses attractive culinary features such as high fiber and low fat contents (Table 16.2). Although the protein content in mushrooms is small when compared to meat, in some developing countries *P. ostreatus* and other edible mushrooms are viewed as the meat of the poor because a diversity of substrates considered waste products are used to cultivate mushrooms under rural conditions, making mushroom proteins more affordable and/or readily available than animal proteins (Gaitan-Hernandez, 2007; Portugal Portugal et al., 2007). Besides its nutritional value, *P. ostreatus* also has antioxidant, antiviral, antimicrobial, antitumor, antimutagenic, antihypercholesterolemic, antihyperglycemic, and hepatoprotective activities (Wasser and Weis, 1999; Iwalokun, 2007; Patel et al., 2012).

16.2 *Pleurotus* spp. as Recyclers

Fungi are one of the few organisms that have the ability to decompose plant organic matter through the secretion of enzymes that break down the components of the plant cell walls (cellulose, hemicellulose, and lignin). In plants, the primary cell wall is composed of cellulose and hemicellulose. Cellulose is a homogeneous polymer of β-(1,4)-linked glucose units while hemicellulose is a heterogenous polymer composed of xylose, mannose, arabinose, and galactose. Lignin, a complex and heterogeneous polymer of aromatic alcohols (Carlile et al., 2001; Webster and Weber, 2007), is found in the secondary cell wall. The enzymes involved in the

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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Table 16.1 Species of *Pleurotus* spp. commercially or experimentally cultivated around the world.

Species	Common or Commercial Name	Distinctive Characteristics Morphological Characteristics	Substrates for Cultivation	References
P. cystidiosus	Abalone mushroom, maple oyster mushroom, Bào Yu Gū	Pilei are small and cream-white in color with strongly decurrent gills. Grow in clusters. Form synnemata on agar cultures.	Straws and hardwood sawdust	Aloha Medicinals, 2015; Guzman et al., 1991
P. cornucopiae	Branched oyster mushroom	Pale cream to pale brown pileus shaped as a funnel.	Straws, sawdust, and other agricultural wastes	Aloha Medicinals, 2015; Buchanan, 1993
P. cornucopiae var. *"citrino-pileatus"*	Golden oyster mushroom, yuhuangmo, tamanoki	The pileus is lemon yellow in color. Fragile basidiomata. Grow in clusters.	Diverse straws	Aloha Medicinals, 2015; Buchanan, 1993
P. djamor	Pink oyster mushroom	It has an attractive pink color.	Barley straw, tea leaf waste with wheat straw, sawdust	Gaitan-Hernandez and Salmones, 1999; Islam et al., 2009, Salmones et al., 2004; Upadhyay et al., 1996
P. djamor var. *salmoneostramineus*	Pink oyster mushroom	Salmon-pink basidiomata. Grows in clusters.	Diverse straws and hardwood sawdust	Guzman 2000; Mora and Martinez-Carrera, 2007
P. eryngii	King oyster, eringi, xìng bào gū, cardoncello, pleurote du panicaut	Medium sized basidiomata with a thick white stipe and pale cream to grayish colored pileus.	Sawdust, rice straw, cottonseed hulls, and grounded corncobs	Moonmoon et al., 2010; Rodriguez Estrada et al., 2009
P. eryngii var. *ferulae*	Eringi	Medium size basidiomata with a light pileus and a central stipe.	Cottonseed hulls	Rodriguez Estrada, 2008
P. eryngii var. *elaeoselini*	Eringi	Medium size basidiomata with a light pileus and a central stipe.	Cottonseed hulls	Rodriguez Estrada, 2008
P. "florida"	Dhengri	Oyster-like shaped pilei.	Soybean, paddy, and wheat straws	Ahmed et. al., 2008
P. nebrodensis	Bailing mushroom	Large fruiting bodies with white caps and stipe.	Cottonseed hulls	La Guardia et al., 2005; Rodriguez Estrada 2008
P. opuntiae	Hongo de maguey	White or cream pileus with an eccentric stipe.	Diverse straws	Mora and Martinez-Carrera, 2007

(Continued)

Table 16.1 (Continued)

Species	Common or Commercial Name	Distinctive Characteristics Morphological Characteristics	Substrates for Cultivation	References
P. ostreatus	Oyster mushroom	Dark gray-brown or blue tinted pileus. Larger than *P. pulmonarius* (pileus diameter is 12–18 cm) with a lateral stipe.	Wheat, barley, oats, rice, rye straw; diverse types of sawdust; cottonseed hulls; and multiple agricultural wastes (coffee, cotton, husks, etc.).	Buchanan, 1993; Guzman et al., 1983; Sanchez and Royse, 2001; Martinez-Carrera, 1989
P. ostreatus var. *columbinus*	Blue oyster mushroom	Pileus with an attractive blue tinted color, especially at immature stages. Grows in clusters.	Rice straw with wheat bran and sawdust	Gibriel et al., 1996
P. pulmonarius	Phoenix mushroom, gray oyster mushroom	Pale colored and lung-shaped pileus with eccentric stipe. Smaller than *P. ostreatus* (<10–13 cm pileus diameter).	Straw and sawdust	Aloha Medicinals 2015; Buchanan, 1993
P. sajor-caju	Dhingri oyster, gray abalone oyster	Brown basidiomata.	Rice straw with wheat bran and sawdust	Aloha Medicinals, 2015; Gibriel et al., 1996
P. sapidus[1]	N/A	Cream colored basidiomata.	Tea leaf waste with wheat straw	Upadhyay et al., 1996
P. tuber-regium	Tiger king mushroom, king tuber mushroom	Develops from dark-brown sclerotia. Cup-like shaped pilei.	Straw, cassava waste, corn cobs, sawdust	Isikhuemhen and Okhuoya, 1996

[1] *P. "florida" and P. sapidus* are invalid species' names. Buchanan (1993) reports that strains of *P "florida"* belong to *P. ostreatus* or *P. pulmonarius. P. sapidus* is a synonym of *P. cornucopiae*.

degradation of lignin, cellulose, and hemicellulose are diverse. On one hand, hydrolytic enzymes break down cellulose while oxidative enzymes, such as laccases (phenoloxidases) and peroxidases (manganese and lignin peroxidases) are involved in the degradation of lignin (Carlile et al., 2001; Mansur et al., 2003). Fungi that can degrade lignin, cellulose, and hemicellulose are known as white-rots because the wood appears white (bleached) after lignin degradation. Fungi that cannot degrade lignin are known as brown-rots because the lignin is left intact and becomes oxidized acquiring a brown, often checked, appearance (Webster and Weber, 2007). *Pleurotus* species are white-rots and therefore suitable to grow on a wide diversity of lignocellulosic substrates that need no previous composting, as in the case of *Agaricus bisporus*.

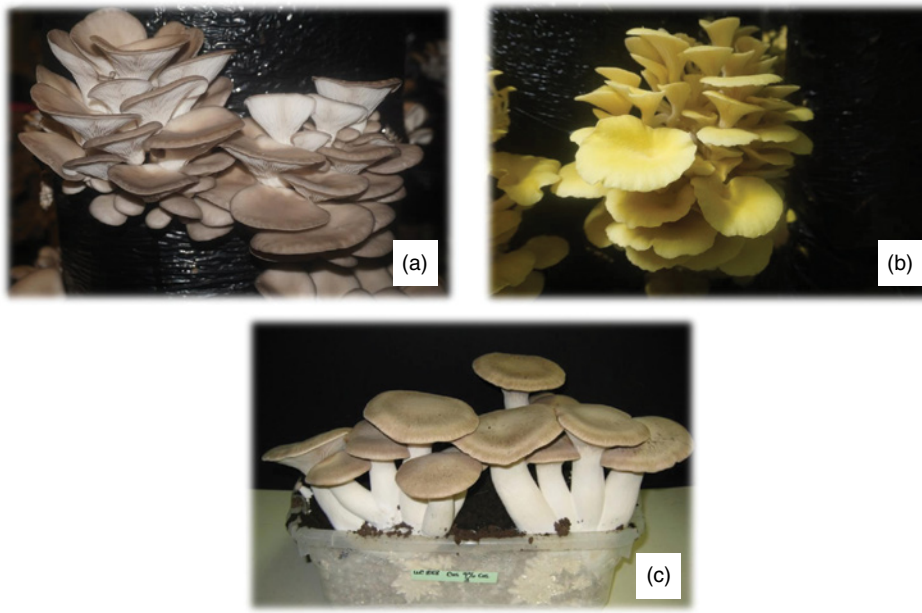

Figure 16.1 (a) Cluster of *Pleurotus ostreatus* basidiomata. (b) *Pleurotus cornucopiae* var. *"citrino-pileatus,"* the golden oyster mushroom. (c) *Pleurotus eryngii* growing on cottonseed hulls substrate. (*See color plate section for the color representation of this figure.)*

Table 16.2 Nutritional composition (fat, carbohydrates, dietary fiber, and protein) of different edible mushrooms (numbers are percent of the fresh weight).

	Moisture	Total Fat	Total Carbohydrates	Dietary Fiber	Total Protein	Calories
P. ostreatus[1]	88.7	0.7	2.6	4.5	1.8	26.6
P. eryngii[1]	89.3	0.7	3.7	3.8	1.3	29.7
Agaricus bisporus[1]	90.7	0.2	0.7	1.9	2.7	15.4
Lentinula edodes[2]	79.8	0.4	17.6[3]	17.6	0.9	72.8
Flammulina velutipes[2]	87.9	0.5	10.6[3]	10.6	0.5	43.4

[1] La Guardia et al., 2005.
[2] Reis et al., 2012.
[3] This value also includes the fiber.

16.3 Cultivation of *Pleurotus ostreatus*

Mushroom cultivation is an activity through which agricultural and forestry residues are recycled to produce nutrient-rich foods. Cultivation of *A. bisporus*, commonly known as the button mushroom, formally started in the eighteenth century in Paris, France. The early development of this technology and its broad culinary acceptance make this species the most cultivated edible mushroom worldwide. Species in the genus *Pleurotus* are the second most cultivated in the world. Although its cultivation did not start until the late twentieth century (Sanchez and Royse, 2001), this genus constitutes approximately 27% of the world production (Royse, 2014). Cultivation of *Pleurotus* spp. started to bloom in the 1970s in central Europe and

North America where it became evident that mushrooms could be grown under controlled conditions over lignocellulosic substrates (Muez Ororbia and Pardo Nunez, 2001). *Pleurotus ostreatus* is often viewed as one of the easiest and most cost-effective mushrooms to cultivate at different commercial and experimental scales. The cultivation of this species starts with the production or purchase of spawn, continues with the preparation of the substrate, inoculation, incubation, primordia formation, mushroom development, and it is completed with the harvesting of mature basidiomata. In the sections that follow, each stage of the production cycle is described.

16.3.1 *Pleurotus ostreatus* Strains (Cultures)

In microbiology, the word strain refers to a genetic variant of a given species. Several strains of *P. ostreatus* are cultivated around the world and there are different ways through which strains of *P. ostreatus* become available to growers: (1) tissue isolation, (2) spore isolation, (3) purchase of cultures from specialized suppliers, and (4) spawn purchase.

1) *Tissue isolation*: *P. ostreatus* is a saprotrophic fungus that grows on hardwood logs during the rainy season (Kay and Vilgalys, 1992). Wild carpophores can be collected from nature, properly identified, and isolated. Pure cultures and normal culture transfers are handled in aseptic conditions, using sterile tools and media. Potato dextrose agar (PDA) is the most commonly used medium. PDA medium is prepared by mixing a commercially available PDA power with deionized water and autoclaving for 15–20 minutes at 121°C and 15 psi. PDA can also be made from scratch using potato infusion (made from boiling sliced potatoes), dextrose, and agar mixed in water. The sterilized media is then poured into petri dishes and allowed to solidify at room temperature. It is advisable that the solidified PDA media is left at room temperature for a couple of days before use. This practice allows two things: (1) water condensed on the interior of the dishes' lids evaporates, and (2) if the sterilization process was not done properly, bacteria or molds will develop on the solidified media and should not be used.
   The culture is obtained by performing a small cut (with a sterile scalpel) on one of the edges of the pileus to crack it open. In this way, the sterile scalpel does not touch the center of the pileus and that area remains clean. With sterilized tweezers, a small piece of tissue is removed (from the flesh) and placed onto the PDA media. Cultures are incubated at ~ 25°C. These cultures should be examined often to make sure they are free of contaminants. Once a pure culture is obtained, it should be prepared for long term preservation in 10% glycerol at –80°C or in liquid nitrogen at –196°C (Lara-Herrera et al., 1998; Kitamoto et al., 2002; Kaur et al., 2011).
2) *Spore isolation*: Isolation of pure cultures from spores is used only if breeding is the objective. Because spores are the result of sexual reproduction, cultures started from spore isolates will be genotypically and possibly phenotypically different from the parent strain. Single spore isolates are obtained from spore prints obtained on sterile paper. A small piece (~5 × 10 mm) of the spore print is placed in sterile deionized water and mixed vigorously. A small aliquot of the spore-water solution (50–100 μL) is placed on the center of a PDA plate and evenly spread with a sterile metal or glass rod. One or two days later, the plates are examined for germination of single spores (SSI, single spore isolate). If found, SSIs are immediately transferred onto new PDA. *P. ostreatus* has a bifactorial (*mat*A and *mat*B) tetrapolar mating system and its spores are monokaryotic. Thus, SSIs are crossed in all possible combinations and compatible mating types are then determined through the presence of clamp connections under the microscope. Larraya et al. (2001) explored the feasibility of using molecular markers to select fast-growing

monokaryons that could result in the development of fast-growing dikaryons, desirable for commercial cultivation.

3) *Purchase from specialized suppliers*: different institutions around the world hold *Pleurotus* spp. strains for commercial or experimental cultivation. Culture collections and suppliers that specialize in mushroom growers' equipment sell previously characterized strains. Cultures are grown on solid nutrient media in slant tubes or Petri dishes and shipped to the costumers. The cost per strain fluctuates between $70 and $350, depending on the supplier and characteristics of the culture. Mushroom growers should be aware that restrictions might apply and special permits for national or international shipping should be arranged in advance.
4) *Spawn purchase*: Commercially available spawn is usually sold in 2.5–20-lb polypropylene bags. Small operations typically purchase spawn in 2–4-lb bags compared to the 20-lb bags produced by one of a few large commercial spawn companies in the United States. Some smaller growers have their own spawn laboratories where they maintain and produce the spawn from starter cultures.

16.3.2 Spawn

In mushroom cultivation, the term *spawn* is used to describe mycelium grown on grain or other substrate that will be used as inoculum for the production of mushrooms. As mentioned previously, the spawn can be acquired from large spawn producers or prepared in relatively small laboratories. The process of spawn production at small scale starts once a pure culture has been obtained. The fungal colony is grown on PDA for one to two weeks before it is transferred to a grain substrate (Figure 16.2). In the United States, rye and millet grains are most commonly used, though wheat, rice, and sorghum can also be utilized. Protocols to prepare the spawn are diverse, but in any case, they share two critical steps: grain hydration and sterilization. Sorghum grain, for example, needs to be pre-soaked for 24 hours in tap water. If the volume of spawn to be prepared is small enough, the sorghum can be boiled for 15 minutes in order to assure grain hydration before the sterilization process. The goal is to assure that the moisture content of the grain is approximately 55% without incurring in-grain smashing or overhydration that could lead to microbial contamination (Guzman et al., 1983). Once the grain is properly hydrated, it is placed in polypropylene bags. The spawn prepared from rye or wheat grain and sawdust follows a different protocol. 100 ml beaker full of rye grain, 50 mL beaker full of sawdust, half a teaspoon of calcium carbonate (lime powder), and 120 mL of warm tap water are mixed in 500 mL glass flasks (Mushroom Spawn Lab, 2015; Figure 16.2). Other containers suitable for spawn production are polypropylene gusseted bags with filters and glass jars. After hydration, the

Figure 16.2 Mushroom mycelium growing on potato dextrose agar (left), partially colonized grain (center) and completely colonized grain (right). Reproduced with permission from the Spawn Laboratory, The Pennsylvania State University.

Figure 16.3 (a) Liquid spawn incubation system in China. (b) Oyster cultivation in small bags inoculated with liquid spawn (China).

grain is sterilized in an autoclave at 121°C and 15 psi for 45 minutes. The grain is cooled down at room temperature and inoculated with agar plugs colonized with *P. ostreatus* mycelium. Incubation of the grain is done in darkness at 20–30°C (depending on the strain). One week after grain inoculation, the grain is vigorously shaken with the purpose of re-distributing the already colonized grain so new inoculation points re-start the colonization process. The mycelium will completely colonize the grain in approximately two more weeks. Although this colonized grain could be used to inoculate the substrate for mushroom production, most laboratories that prepare spawn for mass production will use the first spawn to prepare a second batch. In this case, a small amount of grain will be used as inoculum instead of agar plugs.

Liquid spawn is rarely used in commercial cultivation of mushrooms in North America and Europe though it is very common in parts of Asia (Figure 16.3.a–b). Quimio (2001) described a process where liquid broth contained in glass jars is inoculated with colonized agar plugs, incubated in a shaker incubator for a few days and then ground. Liquid inoculum made of spores rather than mycelium is also commercially available and used in mushroom growing kits for personal use.

16.3.3 Substrate

The substrates and technologies used to cultivate *P. ostreatus* are quite diverse. As per substrates, the choice of materials is determined by its cost, availability, and suitability for mushroom cultivation. Substrates for mushroom cultivation come from agricultural and forestry activities including wastes produced from harvesting and processing of grains (e.g., straws), coffee, sugar cane, and many other crops, sawdust, and so on.

The chemical composition of the substrates used to cultivate *P. ostreatus* is versatile because the C/N ratio can range between 30–300:1 (Muez Ororbia and Pardo Nunez, 2001). The substrates used for the cultivation of *P. ostreatus* are often used as mixtures of two or more components in order to provide the best substrate composition. Usually one substrate will be used as a base material and the rest are used as supplements. Worldwide, straws are among the most widely used substrates containing a high content of lignocellulose and 1–2% nitrogen (Muez Ororbia and Pardo Nunez, 2001). Other substrates used for the cultivation of *P. ostreatus* are sugar cane bagasse, leaves (e.g., banana, coffee, sycamore fig, tea), coffee pulp, sunflower stems,

seed hulls (e.g., cotton, sunflower, peanuts), milled corncobs, etc. (Royse and Schisler, 1987; Alemu, 2013). Because the nitrogen content of most substrates is low (less than 1%), substrates are often supplemented with materials rich in nitrogen such as different types of brans (e.g., wheat, rye, rice, corn) and, in the United States, commercially available delayed-release nutrient supplements.

The design of substrates mixtures is often done based on the availability of the raw materials, chemical composition of the raw materials, and experiences of the mushroom growers. Fernandez-Rodriguez and colleagues (2014) developed an interactive computer program that allows the design of standardized substrate mixtures in order to reach desired C/N ratios, lignin, hemicellulose, cellulose, and mineral content of the resulting substrate mixture. For geographic areas where the availability of raw materials significantly fluctuates, counting with this tool allows growers to quickly design a new substrate mixture with the same desirable chemical composition.

Besides its chemical composition, the substrates should also fulfill physical characteristics related to the particle size and water content. An optimal particle size will prevent water accumulation in the base of the container while, at the same time, prevent substrate compaction in order to allow the mobilization of oxygen, carbon dioxide, water, and metabolic products of the cellular respiration. Thus, particles that range between 2 and 5 cm in length are often used (Muez Ororbia and Pardo Nunez, 2001). Small scale operations can chop wheat straw in bale choppers whereas larger commercial operations typically use equipment such as tub grinders. The raw materials (Figure 16.4) that will constitute the substrates are homogenized dry and then the moisture content of the substrate is adjusted to 65–70%. In order to reach that goal, small facilities place the dry substrates in large metal baskets that are immersed in tanks with water for 20 hours (Guzman et al., 1983). Larger farms might have large mixing tanks where additional supplements and water are added prior to pasteurization. Beside the

Figure 16.4 (a) Chopped wheat straw. (b) Pelletized cottonseed hulls. (c) Tractor about to unload homogenized wheat straw substrate into a pasteurization tunnel. (d) Washed and disinfected tractor used to transfer the substrate from the pasteurization tunnel to the spawning room.

enhancement of nitrogen content, the addition of fine-particle supplements to the substrate (e.g., brans and flours) has the additional benefit of providing finer particles to aid in water retention.

16.3.4 Delayed-Release Nutrients

Schisler and Sinden (1962) demonstrated that protein-rich supplements added to *A. bisporus* substrates at spawning significantly increased yields. However, substrate supplementation so early in the production cycle translated into readily available nutrients that could result in substrate overheating and growth of competitor microorganisms. Later, Carroll and Schisler (1976) developed a method in which oil droplets were encapsulated with a denatured protein coat with the goal of delaying the release of nutrients until the mycelium was well-established in the substrate. In the late 1970s and beginning of the 1980s, the use of delayed-release nutrient supplements in *Pleurotus* spp. began to be explored. In the United States, the addition of commercially available delayed-release supplement is commonly used by large *Pleurotus* farms to increase nitrogen content which boosts mushroom yields.

16.3.5 Substrate Pasteurization

The purpose of pasteurization is the elimination of competitive microorganisms and mushroom pathogens that might interfere with the development of *P. ostreatus* mycelium. The process of pasteurization is done through thermal treatments using hot water or steam. In small operations, the substrate is placed in metal baskets and immersed in water (200 L tanks) at 80–85°C for 30–45 minutes (Guzman et al., 1983). Sanchez and Royse (2001) stress the need to immerse the substrate in hot water once the pasteurization temperatures are reached in order to create a thermal shock and assure the effectiveness of the process. Medium scale operations might utilize cement mixers or specialized straw pasteurization machines that pasteurize, inoculate, and bag the substrate. Pasteurization in enclosed chambers, using injected steam, is better suited for larger operations due to the high volumes of substrate to be processed and the higher costs associated with such equipment. In these systems, temperatures are held between 60 and 100°C for 1–12 h (Muez Ororbia and Pardo Nunez, 2001; Sanchez and Royse, 2001). Some facilities might add fungicides during the pasteurization process in order to prevent substrate contamination (Bermudez et al., 2001).

16.3.6 Alternative Methods of Substrate Preparation

The use of fermented substrates for the production of *P. ostreatus* is usually done in tropical or subtropical regions in small scale, low cost operations. Aerobic fermentation of raw materials is necessary when fresh substrates rich in sugars such as coffee pulp, sugarcane bagasse or banana stems, and so on are used. Simple carbohydrates, such as sugars can promote the growth of competitive molds and bacteria, negatively influencing *P. ostreatus* growth (Guzman et al., 1983). The process of fermentation involves the consumption of soluble simple sugars by bacteria. Since microbial growth and metabolism are actively occurring in fermenting substrate, the temperature of the substrate tends to rise to 60°C for several days. Substrates such as coffee pulp require 3–5 days of fermentation but sugarcane bagasse might require up to 10 days. The substrate is arranged in piles, covered with black plastic and manually turned every several days in order to keep an aerobic environment (Guzman et al., 1983). After the fermentation is completed, the temperature decreases, and the pH is adjusted to 5.5–6.5 by adding calcium sulfate or calcium carbonate (2–4%).

Contreras and colleagues (2004) had reported a low output method to treat the substrate for *P. ostreatus* cultivation consisting of soaking the substrate in alkaline water (pH 8.4–8.5) for different lengths of time. This method is utilized by small scale growers in the state of Chiapas, Mexico. In this method, the raw materials are soaked in tap water (28–29°C) with 5% of commercial calcium hydroxide for up to 48 hours, followed by draining the water, packing, and spawning. Substrates such as grasses, corn straw, corn cobs, and mixtures of those are feasible to use with this method. The pH of the substrate after alkaline water treatment is usually between 8 and 11. Biological efficiencies (BEs) of up to 126% (three flushes) for grass (*Digitaria decumbens*) substrate soaked during 48 hours were reported by Contreras et al. (2004). The disadvantage of this method revolves around the high likelihood of bacterial contamination. Different factors that might influence bacterial populations are: soaking time, pH, and substrate composition. Thus, careful characterization of the substrate and its physicochemical properties might be helpful to prevent contamination.

16.3.7 Substrate Packing

After the process of pasteurization is over, the substrate should be transferred to an aseptic room to be filled into bags and inoculated simultaneously, once the substrate is cooled down to room temperature. The most commonly used containers for *P. ostreatus* cultivation are clear polypropylene gusseted bags with or without filters or black or transparent polypropylene bags. Bags can be purchased with holes pre-punched or the grower can punch small holes or cut slits in the bag to allow air exchange and provide an opening for future pin formation (Figure 16.5). If black bags are utilized, it is a good idea for a few transparent bags to be prepared with each batch of new substrate. Transparent bags allow for monitoring of the mycelium to assure the spawn is colonizing the bags properly and that no contaminant molds are growing in the substrate (Sanchez and Royse, 2001). Although the size of the substrate block is variable, 4–20-kg bags are commonly used, either as cubical, or elongated blocks.

Figure 16.5 (a) Substrate incubation in black polypropylene bags with slits and (b) holes.

16.3.8 Inoculation

Inoculation of the substrate can be done by simply mixing the spawn with the substrate in a ratio of 2–5 % (wet weight) as the bag is being filled. A different method of inoculation consists on filling up the bags with layers of substrate and alternate layers of spawn until the bag is almost full. Bags are either closed with plastic ties, heat sealed, or simply by tying a knot. Whichever method is used to close the bags, the bag should be tied or sealed as close to the substrate as possible to minimize headspace above, preventing mushroom formation on top of the substrate and to prevent moisture lost.

16.3.9 Incubation

Incubation is the phase when the mycelium of *P. ostreatus* colonizes the substrate. In general, this stage is done at 25–30°C, depending on the strain used, in darkness. Five days after the inoculation, polyethylene bags that do not have filters are evenly perforated in the top quarter of the bag with a sterilized scalpel, dissecting needle, or razor blade to allow for gas exchange. The length of the incubation period varies, depending on the *P. ostreatus* strain, and typically ranges from 12–17 days. A complete colonization is achieved when the mycelium has grown, completely covered the substrate, and a compacted layer of white mycelium is visible through the bags.

During the incubation stage, the bags should be closely monitored for mold contamination. Bags showing signs of mold contamination should be removed immediately from the incubation room to minimize mold spread in the production rooms. Several bags showing signs of mold or bacteria contamination might indicate that the pasteurization was inadequate or that the spawn used was contaminated. It is essential to keep detailed records of the substrate and spawn batches used during cultivation in order to pinpoint possible sources of contamination.

16.3.10 Primordia Formation and Development of Mature Basidiomata

Primordia (singular primordium) or pins (Figure 16.6 and 16.7.a) are the first structures that lead to the development of the basidiomata (singular basidioma), commonly known as fruiting bodies or mushrooms. Primordia formation is induced once the substrate is completely colonized with the mycelium and the bags are transferred to a fructification room (if a separate room is available for that purpose). Induction of primordia is done by changing the environmental conditions. The temperature in the room is lowered to 12–22°C, depending on the strain, the relative humidity is increased to 85–90% and a light cycle is implemented (Guzman et al., 1983). In rural conditions, where the cultivation rooms may not have systems to increase

Figure 16.6 Oyster primordia developing from a fully colonized substrate bag.

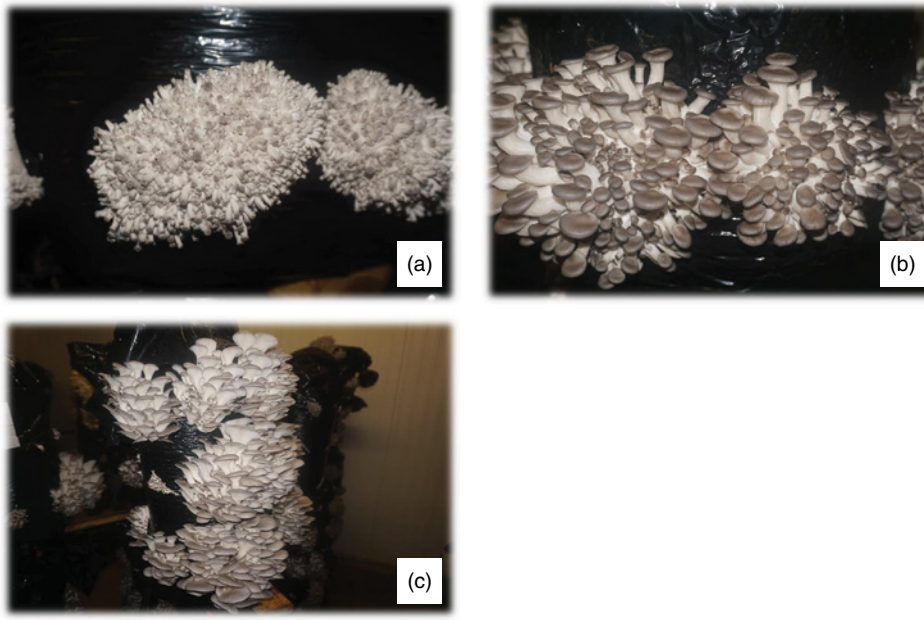

Figure 16.7 (a) Heavy cluster of oyster mushrooms' primordia. (b) Young oyster mushrooms growing on cottonseed hulls/wheat straw substrate. (c) Mature oyster mushrooms growing on cottonseed hulls/wheat straw substrate. (*See color plate section for the color representation of this figure.)*

the relative humidity, mushroom growers water the floor and walls of the rooms daily. If necessary, a gentle watering of the colonized substrate is also done. "V" shape cuttings are added on the surface of the bags to allow primordia formation or around already formed primordia to allow further development onto mature mushrooms. Ventilation within the production rooms is also implemented at this point since concentrations of CO_2 higher than 700 ppm will result in elongated stipes, small pilei, or the complete absence of mushrooms (Sanchez and Royse, 2001).

16.3.11 Harvesting

The mature basidiomata will develop in approximately 5 days after pin formation. Clusters of mushrooms are harvested once the pileus is completely extended but still the margins are rolled inwards, rather than outwards (Figures 16.1a, and 16.7c). The clusters are removed using a knife, carefully and completely removing all the stipe. Leaving pieces of stipe still attached to the substrate increases the chances of mold or bacteria development.

A cropping cycle or flush is a period of conspicuous development of badisiomata. Multiple flushes can be obtained per bag of substrate with the first flush commonly being the most productive. At the commercial level, three flushes are typically harvested before the substrate is discarded. Biological efficiency (BE) is a term commonly utilized to describe how efficiently the substrate has been utilized by the fungus (wet weight of the harvested mushrooms/dry weight of the substrate × 100). At the experimental level, a BE of nearly 180% was reported for mushrooms grown on pasteurized coffee pulp in a rural mushroom farm (Bermudez et al., 2001). At the commercial level, BEs of approximately 100% are typically obtained.

Once harvested, the mushrooms are placed in plastic crates for transfer. Alternatively, mushrooms might be packed for sale to distributors and stores (Figure 16.8). Some facilities that

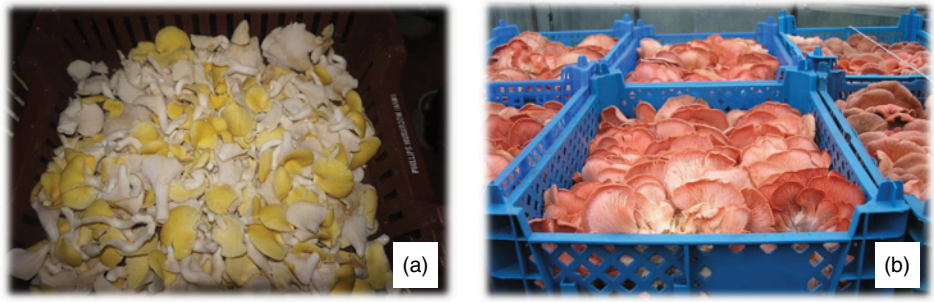

Figure 16.8 (a) Harvested golden oyster (*P. cornucopiae* var. *"citrino-pileatus"*) mushrooms and (b) pink oyster mushrooms (*P. djamor* var. *salmoneostramineus*) in plastic crates.

have their own packing houses may slice or mix oyster mushrooms with other varieties to be sold at grocery stores. The actual packing and shipping procedure varies for each grower.

16.3.12 Spent Substrate

Spent substrate has several potential uses, though most often it is just discarded. There is a considerable amount of usable matter left in the spent substrate and there have been discussions about the feasibility of using spent substrate as a feed source for animal production. Additionally, spent substrate could be added to formulations of *A. bisporus*; however, these practices are not currently incorporated in farms. Additionally, spent substrate could be combined and composted to produce marketable soil amendments.

16.4 Diseases and Pests

As with any other crop, mushroom cultivation is also threatened by pests and diseases especially because the environmental conditions necessary for mushroom cultivation are also conducive to the development of pests, pathogens, or competitive organisms. In most cases, strict hygiene and sanitation can help minimize the development of molds and the incidence of insects in the growing rooms. There are very few effective fungicides and insecticides currently available to mushroom growers. The American Mushroom Institute (AMI, 2014) keeps a list of currently labeled chemicals and biocontrol agents available to mushroom producers. The list is updated as labels are removed or added (http://americanmushroom.org/?page_id=267).

16.4.1 Green Mold Diseases

Although there are more than 30 fungal species (and strains) that might cause green mold disease in *P. ostreatus*, *Trichoderma* spp. are the most common causal agents in mushrooms (Cha, 2004). The genus *Trichoderma* comprises asexual soil-borne filamentous fungi. Different species in the genus are known to grow on *P. ostreatus* substrates: *T. aggressivum*, *T. atroviride, T. virens*, *T. viride*, *T. hazianum*, *T. koningii,* and *T. crassum* (Cha, 2004; Yang et al., 2005). The mycelium of *Trichoderma* spp. is white and difficult to distinguish from *P. ostreatus*. However, *Trichoderma* spp. produces green spores (in conidiophores) and therefore green patches that extend over the *P. ostreatus* mycelium are indicative of *Trichoderma* spp. infections and are easily recognized by mushroom growers. Microscopic observations can also confirm the diagnosis since *Trichoderma* spp. produce hyaline branched conidiophores (asexual reproductive

structures where asexual spores are formed) with one single-celled conidia born in terminal clusters (Barnett and Hunter, 1998).

Trichoderma spp. contamination often results from improper pasteurization of the substrate or during the spawning stage if the spawn was contaminated. If this is the case, the entire batch of substrate will typically present signs of contamination. If only a few bags become contaminated, the cause might be an improper sanitation and disinfection of the spawning room, incubation room, or the tools used during spawning. Flies are important pathogen vectors and should be controlled in order to prevent *Trichoderma* spp. spread within cultivation rooms. Thus, hygiene and sanitation, as part of an Integrated Pest Management Plan (IPM), are critical to keep disease pressures under control.

16.4.2 Dry Bubble

The dry bubble caused by *Verticillium fungicola*, an asexual filamentous fungus, is one of the most significant diseases in *A. bisporus* (Coles and Barber, 2004), but it is only found occasionally in *P. ostreatus*. The colonies of *V. fungicola* are white and the conidiophores are slender with verticillate phialides and ellipsoidal, terminal conidia born singly or in clusters (Barnett and Hunter, 1998). This pathogen can infect the primordia and mature basidiomata. When the primordia become infected, amorphous masses of white and dense tissue develop. If the infections occur once the basidiomata have developed, the mushrooms present cracks and sunken, rotten areas (Gea, 2001). Control of *V. fungicola* through chemicals is not effective. Once the pathogen is present in the crop, it is recommended to remove the infected material since the spores of this species can be easily transported by humans, insects, tools, and water.

16.4.3 Brown Blotch Disease

The organism responsible for most brown blotch diseases in *P. ostreatus* is the Gram negative soil-borne bacteria *Pseudomonas tolaasii*. *P. tolaasii* is known to cause brown blotch in several species of *Pleurotus* and *Agaricus* and the severity of the lesions are bacterial concentration and tissue type dependent. In experimental trials, Rodriguez Estrada and Royse (2005) determined that infections with *P. tolaasii* in *P. eryngii* caused evident lesions at different concentrations (colony forming units, cfu per μL) on the pilei cuticle (5.2×10^5 cfu/μL) and pilei flesh (1×10^5 cfu/μL). Bacteria concentrations below those numbers did not cause disease symptoms.

Brown blotch disease is characterized by brown or yellowish sunken and slimy spots on the pilei (cap) and/or stipe (stem). The spots can expand and completely cover the mushroom. If infection occurs during pinning formation, the basidiomata might not develop into mature mushrooms (Figures 16.9 and 16.10). Besides the obvious symptoms of the disease, *P. tolaasii* could be diagnosed *in vitro* through the *P. reactants* tests that consist of confronting *P. tolaasii* with *P. reactants* on King's B medium. In this test, a white line composed of precipitates is indicative of the presence of *P. tolaasii* strains that produce the toxin tolaasin (Wong and Preece, 1979). The toxin tolaasin is a pathogenicity factor, a low molecular weight lipodesipeptide that induces the formation of ion channels (Brodey et al., 1991). Tolaasin also has biosurfactant properties and is responsible for the observed disease symptoms (Murata and Magae, 1996). Murata and Magae determined that toxin production is associated with bacterial cell density and therefore, as observed for *P. eryngii* by Rodriguez Estrada and Royse (2005), a threshold concentration of the bacteria is necessary to result in disease development.

As in the case of green mold diseases, bacterial brown blotch might be caused by improper pasteurization of the substrate, by the use of contaminated spawn, or by lack of sanitation and disinfection of the growing rooms. Thus, the same preventive measures for green mold diseases apply to brown blotch. Additionally, mushroom growers should avoid excessive air moisture

Figure 16.9 *P. tolaasii* growing on young *P. eryngii* basidiomata.

Figure 16.10 *P. tolaasii* lesions on *P. eryngii* stipes are characterized by striations.

and water condensation over the surface of the mushrooms that facilitates bacterial growth. The regular use of chlorinated water during watering is also a measure to prevent the development of blotch disease (5 ppm) or to arrest its development (20 ppm) (Cha, 2004).

16.4.4 Pink Mold

Pink mold disease is caused by the fungus *Neurospora sitophila* and can often be found growing on *P. ostreatus* substrate bags. *Neurospora*, similar to other fungal pathogens, is associated with contamination that occurs either from contaminated spawn, inefficient pasteurization of the substrate, or through contamination during the spawning and filling process. If infection is severe, yields can be significantly reduced.

16.4.5 Scarids

Lycoriella mali (Figure 16.11.a) is the most important scarid fly that affects *P. ostreatus* crops in North America. The adult flies are important vectors of other pests and pathogens such as mites, nematodes, and molds. Adult flies measure approximately 3–5 mm long and have long antennae (Keil, 2002). Adults lay oval, white eggs in groups of about 100–130 (Cha, 2004). Eggs hatch after 4–6 days of incubation at approximately 20–27°C giving raise to translucent, white larvae (6–12 mm long) that feed from mycelium, destroy pins and mature mushrooms, and create tunnels into stipes and pilei. The larval stage lasts for about 18 days and the pupal stage (2–2.5 mm long) follows for an additional 6 days, depending on the temperature. Once the pupa case breaks, the adult fly emerges and lives between 7 and 10 days. Scarids heavily impact the crop if they enter the cultivation rooms during the incubation stage when the substrate

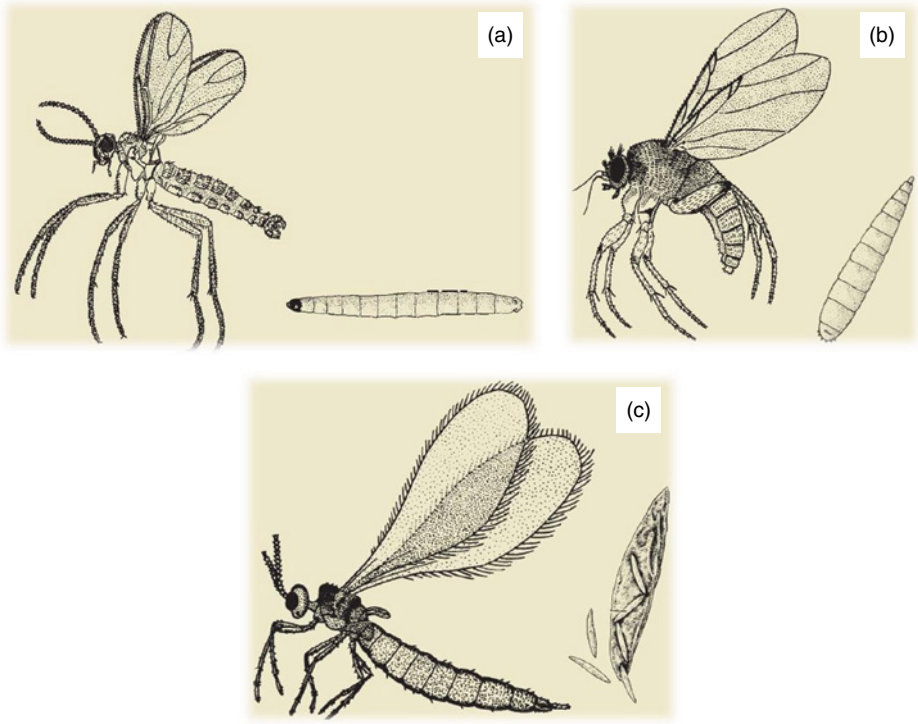

Figure 16.11 (a) Scarid fly, adult fly and larva. (b) Phorid fly. (c) Cecid fly, adult and larvae. *Source:* Reproduced with permission of The Pennsylvania State University.

temperature is around 24°C and the substrate is not yet completely colonized (Gea, 2001; Keil, 2002). Because *L. mali* is adapted to relatively cool temperatures, infestations are less prevalent during the warmer months of the year (Keil, 2002).

16.4.6 Phorids

Megaselia halterata can be numerous in mushrooms crops yet they cause less damage than other flies and therefore it is considered a pest of secondary importance (Keil, 2002). The adult flies measure between 2–4 mm long with small antennae and a characteristic humpback (Figure 16.11.b). Female and male adult flies mate outside the growing rooms. Then, the female flies move to the interior of rooms to lay about 50 white eggs near the incisions made on the substrate bags. The eggs incubate for 2–3 days giving raise to transparent larvae that feed from the mycelium. As the larvae age, their bodies become white and can grow up to 6 mm. The pupae are 3–4 mm long; white at the beginning turning to dark yellow-brown as they mature (Gea, 2001; Keil, 2002). This stage last for approximately 20 days after which the adult flies emerge, reproduce, and usually live for about 8 days.

16.4.7 Cecids

Mycophila speyeri are small flies measuring less than 1 mm (Figure 16.11.c). The larvae are orange and measure 1–3 mm long. As occurs with phorids and scarids, the larvae obtain nutrients from *P. ostreatus* mycelium and also damage the adult basidiomata. This species can dramatically increase its numbers due to a unique mechanism of asexual reproduction called

paedogenic parthenogenesis where the larvae release 10–30 daughter larvae every 6 days. Adult flies are rarely seen in the cultivation rooms (Keil, 2002; Cha, 2004). An inappropriate pasteurization is often associated with infestations by *M. speyeri* and other species of cecids such as *M. barnesi* and *Heteropeza pygmacea* (white larvae).

16.4.8 Springtails (Collembola)

Most springtails belong to the genus *Hypogastrura*. Adult springtails are dark gray to black, jumping insects (1-1.5 mm long) without wings. These insects feed from the mycelium and are often found within the gills of the basidiomata. These organisms favor moist environments and are more common during spring and early summer (Gea, 2001).

16.4.9 Viral Diseases

Viral diseases of *P. ostreatus* occur occasionally. The oyster mushroom isometric viruses I and II (OMIV-I and II) are ds-RNA viruses that cause delays in the formation or complete absence of basidiomata, basidiomata deformations (i.e., shortening of the stipe and minute pileus), and an extreme decrease in growth rate of the infected mycelium (Cha, 2004). It is known that viruses in fungi can spread through anastomosis (fusion of two compatible hypha). Thus, proper sanitation and disinfection of growing rooms where viral diseases are suspected are essential to prevent further infections.

16.5 *Pleurotus* spp. in Biotechnology

Besides the obvious relevance of *Pleurotus* species as edible and medicinal mushroom, other biotechnological applications for this genus are also relevant (Libardi et al., 2012). Many white rot fungi, including *P. ostreatus*, have been studied for their ability to degrade pollutants found in soils. The lignocellulolytic enzymes produced by *P. ostreatus;* lignin peroxidase, manganese peroxidase, versatile peroxidase, and laccase responsible for the breakdown of lignin, are also able to break down soil contaminants such as polycyclic aromatic hydrocarbons (PAH) and benzoprene products associated with fuel spills (Sack and Bunther, 1993; Adenipekun and Lawal, 2012; Bhattacharya et al., 2014; Diaz et al., 2014). Zervakis and Balis (1996) discussed the environmental impact of olive mill wastes in the Mediterranean basin, where the majority of olive tree cultivation and olive oil production occurs. These authors pointed out that *Pleurotus* spp. mycelium can degrade the phenolic compounds present in the dark-brown effluent. In addition, *Pleurotus* spp. can grow on the solid press cake residue with the dual benefit of producing edible mushrooms and further utilizing the spent substrate for animal feed. The ability of *Pleurotus* spp. to pretreat different organic waste products to enhance ethanol production has been demonstrated (Taniguchi et al., 2005; Massadeh and Modalla, 2008). The ability of *Pleurotus* species to degrade phenolic compounds and lignin compounds has beneficial applications to make cellulolytic compounds more readily available to subsequent enzymatic hydrolysis and biological fermentation.

16.6 Future Challenges

In the last 15 years, the demand for readily available lignocellulosic wastes for the production of biofuels and other energy-related applications has become a challenge for mushroom growers, who in the past were able to acquire raw materials at reasonable prices. Thus, mushroom scientists are constantly searching and testing alternative substrates such as office paper,

cardboard, palm (by-product of the flower spathe distillation), tea leaves, and so on. For instance, Mandeel and colleagues (2005) tested the feasibility of paper, cardboard, and palm fiber for the cultivation of *P. ostreatus*, obtaining BEs of 112.4, 117.5, and 95.3%, respectively. Upadhyay and colleagues (1996) tested the feasibility of tea leaf waste for the cultivation of *P. flabellatus* and *P. sapidus* since the animal feed and paper making industry compete for substrates commonly used for mushroom cultivation (i.e., wheat and paddy straw). Spent coffee grounds have recently been used as the main ingredient to grow *Pleurotus* in commercially available mushroom growing kits.

In addition to challenges associated with raw materials supply, the possibility of new diseases suppressing yields is always a constant concern. Some commercial growers have recently reported reduced yields for which scientists have yet to identify the underlying cause. One hypothesis is that the levels of an unknown virus may build up on farms, reducing subsequent crop yields. Mushroom viruses are known to be spread via spores and *Pleurotus* spp. release very high levels of spores during the maturation process.

Though *Pleurotus* species are some of the easiest mushrooms to cultivate, growers can still face many obstacles that might reduce profits. As with any food production system, *Pleurotus* growers must stay abreast of new technologies to keep ahead of future obstacles that can ultimately determine the success or failure of their operation.

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17

Characteristics, Production, and Marketing of the Sun Mushroom: The New Medicinal Cultivated Mushroom

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17.1 Introduction

The "Cogumelo do Sol® = Sun mushroom" has aroused great interest in the scientific community mainly due to the following factors: (1) its medicinal and pharmacological properties, highlighting the high content of β-glucan, in addition to other amino acids and bioactive compounds such as agaritine; and (2) the method of production by traditional composting with the absence of sawdust and the use of local agro-industrial waste in each region. Some researchers have referred to it as a medicinal mushroom, *Ganoderma lucidum*, which differs mainly due to the method of substrate production, for *G. lucidum* sterilized sawdust (as described in Chapter 18), the period of forming the mushrooms, and also to the morphological characteristics of the mushrooms. The Sun mushroom is a fleshy fungus, while *G. lucidum* is a dried mushroom, which hinders culinary consumption. It should also be noted that *G. lucidum* has been grown for a long time, while the Sun mushroom has been grown for just a few decades, contributing to more literature available to the scientific community on *G. lucidum* production.

As for culinary use of the Sun mushroom, there are two opinions: one places it as "unique and different" because of its pronounced flavor similar to almonds, and the other, those who find the smell unpleasant, limiting its culinary use, and view them as only useful as a therapeutic or medicinal product.

In Brazil, the Sun mushroom is popularly known also as "Cogumelo do Sol®", "Cogumelo Medicinal" and "Champignon do Brasil", in the United States as almond mushroom or almond Portobello and Royal Sun Agaricus, in Japan as Himematsutake and Agarikusutake, and finally in China as Song Ji Rong (Amazonas, 2004; Firenzouli et al., 2007). The Sun mushroom was formerly known by the scientific name of *Agaricus blazei* Murrill (sensus Heinemann). In last few years, several articles have focused on the clarification of its botanical name (Wasser et al., 2002, 2005; Kerrigan, 2005, 2007) and agreed the former name was wrong (Largeteau et al., 2011). By studying other specimens in addition to the cultivated strains, two names have been proposed: *Agaricus subrufescens* Peck or *Agaricus brasiliensis* "Wasser et al." (Largeteau et al., 2011). In this chapter, we refer to this species by the name popularly used in Brazil: the *Sun mushroom*.

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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According to the literature, the first known appearance of this basidiomycete in Brazil was in the town of Tapiraí (São Paulo, Brazil), in the mid-1960s, when it still belonged to the province of Piedade (São Paulo, Brazil). An agronomist named Takatoshi Furumoto, who worked, studied, and produced mushrooms (Shiitake and *Agaricus bisporus*), picked it near his farm, in the mountainous region of the Atlantic Forest, also in the state of São Paulo. Furumoto cloned the mushroom and began to do some production testing with this fungus. Cultivation in Brazil started in 1980.

Some samples were sent to Japan to study its medicinal properties and at the time some positive responses were observed following consumption. Studies on taxonomy, morphology, and its medicinal and agronomic properties began to be carried out by the world scientific community. In 1985, after the death of Furumoto, the cultivation of the Sun mushroom in Brazil was forgotten, but in the middle of the 1990s, genetically improved strains (work from the Iwade Institute, Japan) were sent back to Brazil, for better weather conditions. Since then commercial crops have been raised out in traditional ways as well as in industrial production (large scale). It is believed that Asian countries such as China, Korea, Taiwan, and the United States also received some of these strains for cultivation, also producing the Sun mushroom in commercial crops. Definitively, the cultivation of the Sun mushroom has been recent, so there is little knowledge about their production technology – having been developed in different places – and adaptations of the technology used in the production of *Agaricus bisporus* (button mushroom).

17.2 Morphological Characteristics

The Sun mushroom sporophore has, on average, a pileus 3–9 cm in diameter when closed, is semiglobose after opening and truncated at the top, and light brown to cream with a small white scaly protruding top hat (2–3 mm). The stipe narrows at its junction with the pileus, is 4–13 cm long, and 1–4 cm in diameter, with a uniform thickness or a bulbous white base (Figure 17.1).

The lamellae are arranged very close to each other, without connecting the stipe, 0.8–1.0 cm wide, and white to gray brown (dark brown in senescence). Spores are elliptical (4.9–6.4 by 4.0–4.7 μm), dark brown color, thick-walled, nonporous; tetrasporic basidia, and claviform or

Figure 17.1 (a) Morphological aspects of the mushrooms, (b) spore print, and (c) elliptical spore. (*See color plate section for the color representation of this figure.)*

spear-shaped cystidia (18–27 by 6–10 μm) or catenulates with globose elements (5–7 μm in diameter), hyaline, or brown colors (Heinemann, 1993).

17.3 Spawn Production

Spawn production is developed following the steps proposed by Zied (2008): production of subculture → production of mother spawn → production of grain spawn → production of inoculum or spawn.

For the production of the subculture, a culture medium, obtained from 60 g of compost and 1 liter of distilled water, to which 15 g of agar is added, is used. These culture medis are transferred to petri dishes, where subculture is inoculated with an internal fragment of the selected mushroom transferred. Petri dishes after inoculation are incubated at 28 °C for 8 days. Later, after the development of the mycelium, the disks of subculture are transferred to the other petri dishes, named the mother spawn, and incubated at 28 °C for 8 days, using the same culture medium in subculturing.

The production of grain spawn and the spawn occurs on cereal grains (wheat, oats, or triticale). To produce the substrate, the grains are boiled for 40 minutes, subsequently 20 g of calcium carbonate and 50 g of gypsum (slow drying) is added per kilogram of cooked grain. This substrate is transferred to a glass bottle, with autoclaving for 2–4 hours (depending on the size of the bottle), to 121 °C.

For production of grain spawn in the bottles, the mother spawn is divided into eight equal triangular parts (pizza style wedges); each of the parts is transferred to the underside of the bottles placing the substrate (grains) on the mother spawn part, and incubated at 28 °C for 12 days.

Finally, the substrate used for the production of spawn is the same as that used in the production of grain spawn; the only difference is that the grains (±1 kg) are placed in plastic bags (HDPE or PP). Thus, portions of the colonized grain spawn (±6 g) are transferred to the spawn autoclaved substrate (at 121 °C for 2–4 h). Subsequently, the plastic bags are sealed and incubated for 18 days at 28 °C.

Currently, there are no rules on abbreviations or names of the strains (variety) used by laboratories. Thus, each laboratory cloning the specific strain of interest and naming it considers what is most appropriate for identification. Brazil still has not made any genetic improvement to the strains; what is done is a process of cloning to adaptation of the strains, according to the specific conditions of each region or type of installation (chamber of production). In 2011 in Arcachon (France) the first results of research using a hybrid were presented, but still nothing commercially (Zied et al., 2011).

In the Centro de Estudos em Cogumelos of the Facultad de Ciencias Agrárias e Tecnológicas, Universidade Estadual Paulista (UNESP, Câmpus de Dracena), Brazil, there is a large collection of strains characterized by agronomical performance. Table 17.1 shows the results of yield obtained by five strains inoculated into three types of compost (made from crushed sugar cane with Massai, oats, and Aruana; see the scientific names under the heading compost).

This table shows the high yield potential of the ABL 99/30 strain; on the other hand, note the small size of the collected mushrooms and the need for low temperature fruiting (±23 °C), well below the other strains (28 ± 2 °C). The ABL 03/44 and 04/49 strains have a slightly lower performance than ABL 99/30, but the mushroom size is bigger, so they are sold at a higher price due to the marketing standards in Brazil. The ABL 07/59 strain has low yield and high susceptibility to attack of *Lecanicillium* and *Mycogone*, but is earlier (60 days of cultivation).

Table 17.1 Yield (kg mushroom/12 kg of compost) obtained for five strains of Sun mushroom in three different composts.

	Strains of Sun Mushroom				
Yield (kg)	**ABL 99/28**	**ABL99/30**	**ABL 03/44**	**ABL 04/49**	**ABL 07/59**
Massai	1.31 A ab	1.86 A a	1.56 A a	1.29 A ab	0.32 A c
Avena	0.93 A b	2.43 A a	1.19 A b	1.14 A b	0.35 A c
Aruana	0.66 B b	1.49 B a	1.14 A a	1.23 A a	0.20 A bc

Uppercase letters compare the results of the same column; lowercase letters, results of the same line; the different letters differ by Tukey test (5%).

Yield differences are also observed when comparing different substrates. For example, high values are in Massai compost (in the case of ABL 99/28, 03/44, and 04/49 strains) and Avena (with 99/30 and 07/59 strains ABL), because their physical and morphological characteristics (hairiness, number of waxes, etc.) influence the end of the composting process, the degree of decomposition (which sets the amount of macro and micronutrients that will be available to fungi), and the number of thermophilic microorganisms and actinobacterias.

All research related to the Sun mushroom began around 1986, by the late Professor Augusto Ferreira da Eira after scientific mushrooms production in the Módulo de Cogumelos in the Faculdade de Ciências Agronômicas belonging to the Universidade Estadual Paulista (UNESP, Botucatu), to which we owe sincere thanks.

17.4 Compost (Phase I and II)

Compost production for the Sun mushroom is not yet standardized, but there are differences between it and the composting process used in the production of *A. bisporus*, especially regarding the initial formulation of the compost.

The formulations of classical compost, made from horse manure and chicken manure; and composts without manure that have a more stable nitrogen source, are also used in the cultivation of this mushroom. Currently, about 60% of Brazilian growers have changed the classical compost to compost without manure, "synthetic," due to the scarcity and high prices, and the variation in composition and number of physical contaminants normally associated with these materials (plastic bags, paper, stones, etc.). Currently, meals and cakes as an organic nitrogen source are used.

The most commonly used materials for the formulation of compost are:

- Bagasse sugar cane (*Saccharum officinarum*).
- Sugar cane straw.
- Grasses: *Brachiaria* sp., Tifton (*Cynodon dactylon* var. Tifton), Massai (*Panicum maximum* cv. Massai), Coast cross (*Cynodon dactylon*), Aruana (*Panicum maximum*), and so on.
- Manure: horse and chicken.
- Cereal straws: wheat, oats (*Avena sativa*), and rice.
- Meal: soybean, wheat, corn, cotton, rice, and so on.
- Cakes: cotton, peanut, manioc, and so on.
- Synthetic compounds: urea, ammonium sulfate, and single superphosphate.
- Acidity regulators: calcium carbonate, calcite lime, and gypsum.

Table 17.2 shows the formulation of various composts used in the production of the Sun mushroom. The C/N ratio of the compost at the beginning of phase I is 37/1 (Kopytowski-Filho, 2002), while at the time of inoculation is 25–27/1 (end of phase II). The inorganic/organic nitrogen ratio is 0.4–0.6 and the amount of nitrogen may range from 1.15 to 1.45%.

It is noteworthy that, in Europe, various research has been carried out using the same compost initially developed for the production of *Agaricus bisporus* (described in Chapter 6) and this has also obtained high values of yield. In Europe, the only company working commercially

Table 17.2 Formulation of Sun mushroom compost, for the final production of 15 tons; first and second formulation "without manure," and third formulation classic compost.

First Formulation	**Moisture (%)**	**Wet Weight (kg)**	**Dry Weight (kg)**	**C Content (kg)**	**N Content (kg)**
Sugar cane bagasse	35	7000	4550	2376	19.11
Brachiaria sp.	9	2000	1820	910	9.10
Soybean meal	8	550	506	227.7	35.4
Urea	0	50	50	—	22.5
Ammonium sulfate	0	50	50	—	11.5
S. superphosphate	0	100	100	—	—
Gypsum	0	240	240	—	—
Calcite lime	0	400	400	—	—
Total Weight		10,390	7716	3513.7	97.61
C/N Ratio – 36/1			**% N – 1.31**	**N inor/org relation – 0.53**	
Second Formulation	**Moisture (%)**	**Wet Weight (kg)**	**Dry Weight (kg)**	**C content (kg)**	**N content (kg)**
Sugar cane bagasse	35	7000	4550	2247	18.20
Oat straw	11	2500	2225	938	21.14
Soybean meal	8	400	368	165	25.76
Urea	0	40	40	—	18.0
Ammonium sulfate	0	45	45	—	10.3
S. superphosphate	0	100	100	—	—
Gypsum	0	240	240	—	—
Calcite lime	0	400	400	—	—
Total Weight		10,725	7968	3350.0	93.4
C/N Ratio – 36/1			**% N – 1.31**	**N inor/org relation – 0.53**	
Third Formulation	**Moisture (%)**	**Wet Weight (kg)**	**Dry Weight (kg)**	**C content (kg)**	**N content (kg)**
Sugar cane bagasse	40	7000	4200	2100	29.4
Oat straw	11	1500	1335	563	12.6
Aruana straw	9	1000	910	449	5.7
Chicken manure	30	600	420	142	6.3

(Continued)

Table 17.2 (Continued)

Third Formulation	Moisture (%)	Wet Weight (kg)	Dry Weight (kg)	C Content (kg)	N Content (kg)
Soybean meal	8	150	138	62	9.66
Urea	0	40	40	—	18.0
Ammonium sulfate	0	40	40	—	9.2
S. superphosphate	0	100	100	—	—
Gypsum	0	240	240	—	—
Calcite lime	0	250	250	—	—
Carb. Calcic	0	300	300	—	—
Total Weight		11,220	7973	3316.0	90.86
C/N Ratio – 36/1			**% N – 1.31**	**N inor/org relation – 0.53**	

with the Sun mushroom compost on a large scale is the CNC Exotic Mushrooms (Gennep, Netherlands).

Phase I composting is done in facilities with open sides, a cement floor (aerated or not) and a plastic or corrugated iron roof. The production process for the compost follows the traditional method, also called cords or batteries, in which the piles of compost have dimensions of 2 m long and 2 m high, varying the length depending on the final amount desired by each grower. Within the composting process (phase I), the pre-wetting of grass straws with bagasse is made according to the traditional system. Phase I in an airy bunker is rarely seen in Brazil, but is used, following the same practice of *A. bisporus* (Chapter 6).

The frequency of turnings, manual or semi-mechanized (Figure 17.2) is determined according to the following factors: humidity, raw material used (physical structure), and type of patio floor (aerated or not), as well as others. When deciding the best time for filling the tunnel, composting indicators such as temperature, the degree of compost degradation, and the number of actinomycetes and thermophilic microorganisms are considered, because pasteurization is carried out by its own thermogenesis of microorganisms, and is poorer in N content those

Figure 17.2 On the left, a machine used to turn the compost (semi-mechanized, it still needs some manual labor); right, manual turning made in smaller, family farms.

Table 17.3 Operations to be carried out during composting (first formulation).

Day	Process
01	Wetting of the *Brachiaria* and sugar cane bagasse (cord mounting).
04	1st turning of compost and adding water.
07	2nd turning of compost and adding soybean meal and water.
10	3rd turning of compost and addition of water and additives [a)].
13	4th turning of compost and adding water.
15	5th turning of compost and adding water and gypsum.
18	Last turning and addition of water.
21	Filling the pasteurization tunnel.
32	End of phase II composting and inoculation.
e.g., Water is added to the compost daily, to correct the moisture to 65% humidity.	

a) Urea, ammonium sulfate, simple superphosphate, and calcite lime.

used in the production of *A. bisporus* substrate. Some growers also have a boiler to help conduct phase II compost.

Table 17.3 describes the composting process conducted in a commercial crop, from mounting of the cords until inoculation of the compost (end of phase II). This scheme should not necessarily be followed, but it is useful from an educational point of view for understanding the process.

Phase II compost consists of the process of pasteurization at 58 ± 1 °C for 10–24 hours, an aeration of 180–240 $m^3\ t^{-1}\ h^{-1}$ (rate of new air recycling 10–40%) and conditioning at 47 ± 2 °C for 6–9 days, and aeration of 140–200 $m^3\ t^{-1}\ h^{-1}$ (rate of recycled air 5–35%). The facilities used in this phase are tunnels similar to those used in the cultivation of button mushroom.

A good composting process (Phases I and II), represents 60% of the successful cultivation of the Sun mushroom. Other key factors are inoculum or "spawn" high-quality, adequate facilities and casing layer, correct induction of pinning, and control of pests and diseases (a critical factor in this crop due to the high temperatures reached during production).

17.4.1 Other Methods for Compost Production

Some alternative methods have been studied by the academic community, with work on axenic substrate production and inoculation of microorganisms in the compost. The axenic production uses crushed raw material as sugar cane bagase, crushed grass, meal, and calcium carbonate. The initial formulation of the substrate has a C/N ratio between 35–16/1 and, as mentioned previously, the calcium carbonate is used to raise the pH to 6.5–7.0. The moisture content is adjusted to 65% and the substrate is placed in high density plastic bags (HDPE) at a rate of 2.5–3.0 kg wet substrate, being subjected to a sterilization process at 121 °C for 2 h. The method of inoculation of microorganisms into the compost aims to reduce the time to compost and allows small farmers to complete the process on a small scale (amount of compost inferior than 5 kg), where the generation and multiplication of certain thermophiles and beneficial microorganisms in the composting process can be difficult. Some works have been published on this issue, which check the best time to add microorganisms (phase I and phase II) and their influence on yield (Figueiredo et al., 2013; Souza et al., 2014).

17.5 Spawning and Mycelium Run

The amount of spawn used for compost inoculation varies between 0.8 and 1.5%, relative to the wet weight of the compost. Most of the time, Sun mushroom cultivation takes place in plastic bags with 10–12 kg of substrate, but can also be done in plastic boxes and on shelves (70–90 kg per m^2). The amount of spawn used can positively influence yield, the use of a maximum of 3% at the level of commercial production is recommended, since a higher value significantly raises the cost to the grower. Significantly, the better distributed the spawn in the compost, the faster the incubation phase, and lower the possibility of contamination

The ideal mycelia growth temperature is 28 ± 2 °C; with this temperature, the process takes about 12–15 days. This temperature and this time are common for most Brazilian growers. Figure 17.3 shows a greenhouse used for mycelium run in compost. Bags with inoculum or "spawn" should not be sealed, but folded to allow the application of an insecticide (pyrethroid or other) on the bags to prevent the entry of flies and mites, otherwise it comes into contact with the compost.

Due to lack of information and technical assistance, few growers produce compost but most buy it. Colonized compost with mycelium of the Sun mushroom has a whiter coloration than the colonized compost of *A. bisporus,* because the density of mycelium develops easily in the compost (Figure 17.4).

Commerical production of the compost in Brazil is made with colonized compost using gray, beige, and transparent bags. Black color bags are not used because they overly warm the compost and can damage the developing mycelium, especially when transported over long distances by trucks.

17.6 Casing Layer

For the preparation of the casing layer, mineral soil (75% of total volume) is used as the base material. During the period 2005–2006, a study was performed to determine which were the most used materials in the casing layer for the cultivation of the Sun mushroom in the province of Piedade (São Paulo). The most used materials were mineral soil (50%), mineral soil + coal (37.5%), and mineral soil + carbon + vermiculite (12.5%). With respect to the correction applied to the casing layer, 75% of calcium carbonate, 12.5% calcitic lime, and 12.5% dolomitic lime

Figure 17.3 On the left, disposal of compost on wooden shelves for mycelia growth; right, detail of the folded bags that allow the application of insecticide.

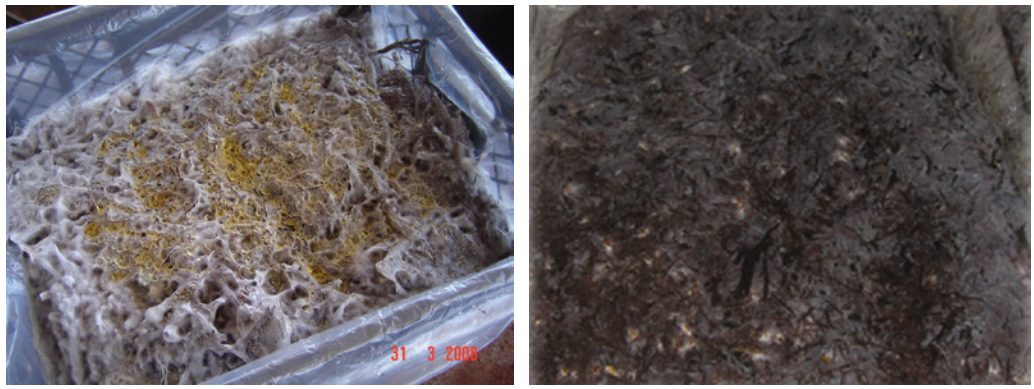


Figure 17.4 On the left, compost colonized by Sun mushrooms; right, compost colonized by *A. bisporus*.

were used by growers to achieve a pH of 6.5–7.0 (Andrade et al., 2006). Due to the high use of mineral soil in the casing layer, Zied (2008) studied the physical, chemical, and microbiological characteristics of the different types of soil. The author emphasizes that for high yield to be obtained, the soil should have approximately 556 g kg^{-1} of sand, 102 g kg^{-1} of silt and 342 g kg^{-1} clay, density 1.0–1.1 g cm^{-3}, water holding capacity of 40–50%, Al + H $mmol_c$ less than 15 dm^{-3}, and a value of base saturation above 70%.

This soil is mixed with other materials, which reduces the density and compaction of the casing layer caused by daily irrigations, and increases the porosity and water holding capacity. Table 17.4 shows the values of yield and the number and weight of mushrooms obtained from four casing layers: mineral soil + charcoal, mineral soil + composted pine bark, mineral soil + coconut fiber, and mineral soil + peat, always in 3:1, v:v.

The casing layer added to the pine bark was the one that had a higher yield, followed by casing based on charcoal, peat, and coconut fiber. However, they emphasized two important factors: (1) from an economic point of view, highlighted the casing layer with charcoal added, due to the low cost of this waste in the state of São Paulo and in Brazil, in addition to high yield observed; (2) the mushroom harvested in the casing layer with coconut fiber has extraordinary physical characteristics for exportation, overlooking the mushroom (which follows a pattern; see the section 17.9 on post-harvest and marketing).

We must emphasize that the mineral soil used in cultivation must go through a process of correcting the pH to a value of 6.5–7.0. The use of a calcium source with low magnesium, such as calcium carbonate or calcitic lime, is recommended. A correcting operation is performed

Table 17.4 Values of yield (kg of mushrooms/12 kg of compost); unit weight of mushrooms (yield divided by the number of mushroom), and number of mushrooms (collected in 12 kg of compost).

	Casing Layer with Mineral Soil (3:1, v:v)			
Variables Analyzed	**Charcoal**	**Peat**	**Pine Bark**	**Coconut Fiber**
Yield (kg)	1.833 a	1.656 a	1.923 a	1.492 a
Weight of mushroom (g)	19.51 b	20.43 b	17.64 b	22.14 a
Number of mushroom (u)	93.96 a	81.0 a	109.29 a	67.39 b

Different lowercase letters in the same line show significant differences in the Tukey test (5%).

5 days before the application of casing over the colonized compost (see results obtained in Exp. 1, later in Figure 17.9).

When casing used consists of mineral soil and charcoal is usually not a mandatory phytosanitary treatment (performed with steam or formaldehyde and other fungicides), especially when the soil is removed to 2 m deep, far from of rhizosphere effect of plants, and comes from regions where there is no large-scale commercial mushroom cultivation. However, when we add materials rich in organic matter and many macro- and micronutrients are easily assimilated, prior phytosanitary treatment of the casing layer is recommended. The phytosanitary treatment may be:

- Physical: steam is used for 5–7 hours at 60–65 °C. Moistening the casing layer before starting treatment (to improve heat transfer) is recommended.
- Chemical: wetting the soil, the casing layer is piled up to 25 cm in height, and with the help of a broom handle, some holes through which a solution of 1 liter of formaldehyde is introduced into 10 liter of water in approximately 1 m^3 of material to be disinfected; after which the material is kept covered with black plastic for 4–5 days.

For both processes 2–3 days are recommended after treatment before using the casing layer.

Although Brazilian growers use mineral soil as a main component of the casing layer, this is not specifically required. Pardo-Giménez et al. (2013) demonstrated that the use of casing layers based on peat (Euroveen and Infertosa) may potentiate yield of the Sun mushroom when compared to mixtures based on mineral soil and other organic materials. The authors also mention that the moisture content of fruiting bodies is affected by the type of casing used.

The thickness of the casing layer to be applied to the colonized compost must be established according to culture conditions of the production room, so that in environments with controlled temperature, the relative humidity and a level of CO_2 coverage is recommended at 3–3.5 cm; in rustic environments with partial control of variables between 4.5 and 5.5 cm is recommended. It should be noted that irregularities in the casing layer as a result of the harvest must be corrected by filling the holes with additional casing material.

A detailed study by Zied et al. (2012) shows the possibility of predicting yield, biological efficiency, earliness, and precociousness based on the chemical attributes of the casing layer, which further reinforces the importance of chemical analysis of the casing layer for an adequate cultivation plan for farmers.

17.7 Facilities used in the Production Process

In Brazil, 45% of growers use greenhouses (with milky plastic film or Duplalon® double-sided "black/white" plastic film) for the production of Sun mushrooms, followed by aligned "Tetra Pak" barracks (45%), outdoor cultivation (6%), cultivation in climatic chambers (3%), and production in rustic structures in forests (1%). Figure 17.5 illustrates the facilities used in Brazil. The only environment fully controlled is a climatic chamber; greenhouses and "Tetra Pak" barracks have only partial control of humidity and temperature.

Due to the current climate change, we cannot expect classic climatological behavior in Brazil (cold season = 17 ± 5 °C and hot season = 29 ± 4 °C), so some growers are seeking public and private funding to modernize their facilities. However, this is on a small scale, even though the product acquires high prices at certain times of the year when the cold season results in low yields. Greenhouse cultivation is still popular in Brazil because of the favorable environmental conditions for production of this mushroom. According to the facilities and the time of year, the established production method for cultivation is boxes or plastic bags (10–14 kg of

Figure 17.5 Facilities used in the production process: (a) greenhouses; (b) aligned "Tetra Pak" barracks; (c) outdoor; (d) controlled chambers; and (e) rustic structures in forests.

compost) or shelves (50–100 kg of compost per m^2). Facilities initially designed for the cultivation of *A. bisporus* and *Pleurotus ostratus* var. Florida can also be used.

17.8 Pinning and Harvest

Pinning occurs, on average, 23 days after the addition of the casing layer. In the cultivation of Sun mushrooms the operation of ruffling is not usually carried out, nor conventional applications of insecticide and fungicide after the addition of the casing layer and along the crop, since this mushroom is used for medicinal, therapeutic, and pharmacological purposes.

Table 17.5 shows the recommended environmental conditions for the development of the crop cycle. In crops in greenhouses and aligned barracks (which have limited control over

Table 17.5 Recommended environmental conditions for the cultivation of Sun mushrooms after casing application.

Environmental Conditions	12 Days After Casing	Fruit Body Induction	During Flushes	Interval of Flushes
Compost temperature °C	27 ± 2	20 ± 2	27 ± 2	28 ± 2
Air temperature °C	25 ± 2	18 ± 2	26 ± 2	27 ± 2
Relative humidity %	90 ± 2	85 ± 2	82 ± 2	85 ± 2
CO_2 content ppm	≥1800	±800	±600	±600

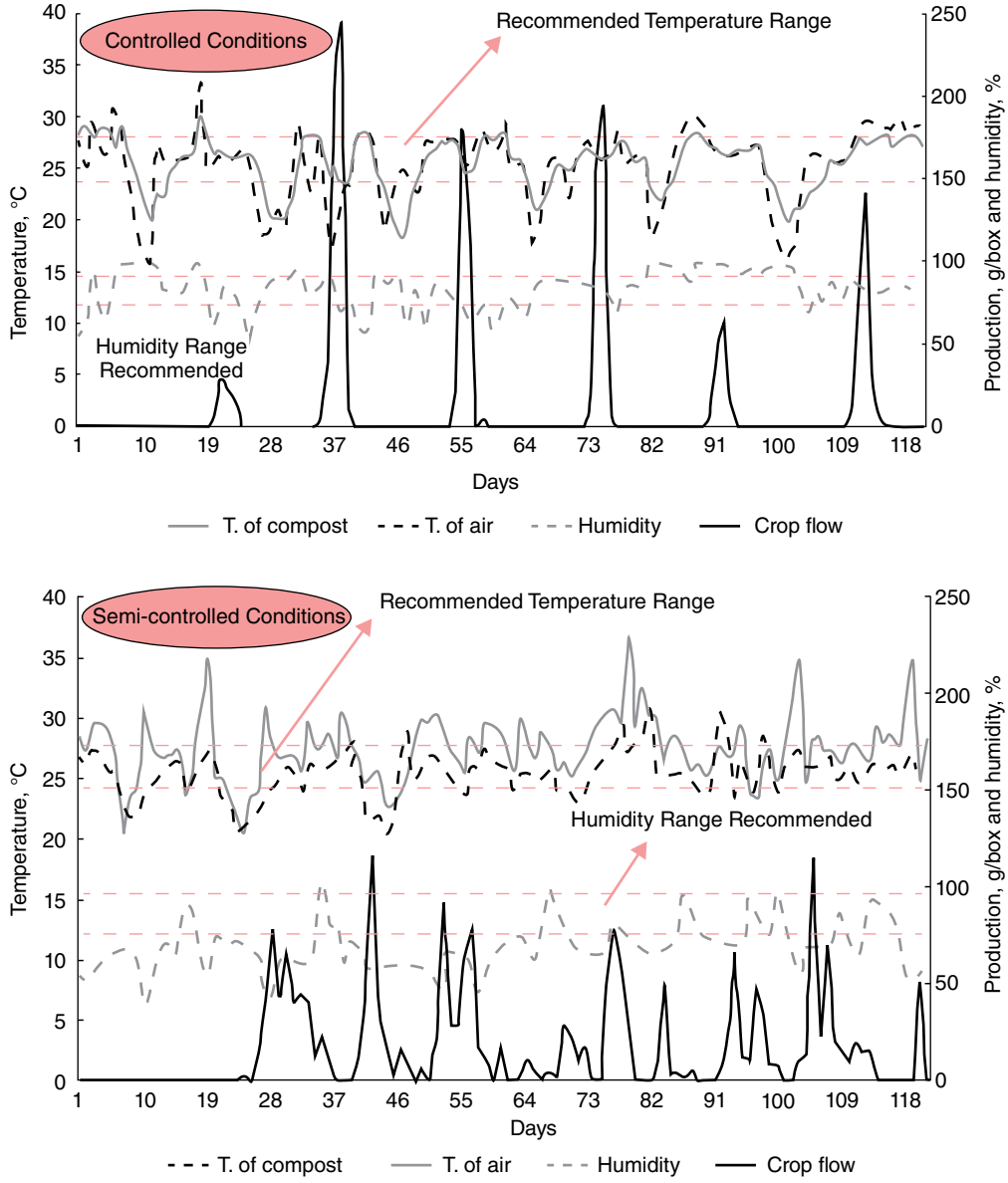


Figure 17.6 Scheme of 120 days production, under controlled conditions (climate chambers), and semi-controlled (greenhouses and aligned barracks), depending on the temperature of the compost, the air (°C), and relative humidity (%).

environmental conditions), the induction of primordia for each flush is at the expense of external environmental conditions.

In cultivation in controlled chambers this does not occur and six flushes of harvest in a production cycle (120 days; Figure 17.6), with three production cycles per year can be achievd. In cultivation in greenhouses and aligned barracks three production cycles per year (with an undetermined number of flushes) are also achieved, although one of them has a low yield. Figure 17.6 shows the behavior of the cultivation depending on environmental conditions, in a

Figure 17.7 On the left, mushrooms with a natural color on the surface of the pileus; right, mushrooms with the "burned" pileus, as indicated by the arrows.

controlled and in a semi-controlled environment (120 days of production after the application of casing).

For cultivation under controlled conditions, the initial temperature 27 ± 2 °C is maintained for approximately 12 days. Then the ventilation is increased and the CO_2 content and temperature of the compost (2 °C per day) are reduced; 16–17 days after the addition of the casing, the temperature reaches ±20 °C, staying there for 48 hours. Subsequently, the temperature of the compost rises 2 °C per day, until it reaches ±27 °C (when the presence of some fruiting bodies is observed). After harvesting it is recommended that the temperature of the compost remain at 28 °C for 7 days, then again reduce the temperature by 2 °C per day, for a new production flush.

In a semi-controlled environment, it is difficult to drive a flush intentionally; they take place according to external environmental conditions. In Figure 17.6, note that as long as there is a reduction in the temperature of the compost of approximately 5 °C, the flush of crop occurs, as evidenced in Figure 17.6 at 23, 40, 54, 69, 95, and 102 days after the addition of the casing layer. Sometimes, under these conditions, the crop can be performed daily; this differs from the harvest under controlled conditions where there are six well defined flushes, each of 5 days' duration, totaling 30 days harvest, for a total of 120 days of production.

When weather conditions are unfavorable (cold temperature and low relative humidity), the grower should be very careful with the temperature of the water used for irrigation of the casing. In Brazil heat treatment of water to around 25–28 °C (ideal temperature for use) may not be done, and growers may use cold water (±17 °C) in mushroom beds, which results in a reduction of mycelia growth in casing and the "burning" of the top of the pileus (leading to mushrooms with a beige to dark brown color (Figure 17.7)); the effect of using cold water is even more pronounced because in this season the relative humidity is very low.

We are currently developing a new methodology for primordia induction and conduction of crop cycles in controlled environments, called a "fast cooling method" (Figure 17.8), with incubation with a compost temperature of 20–28 °C, drop for fruiting and compost temperature of 26 °C and 700 ppm CO_2 during the cultivation. The difference in the method shown previously is the fast reduction for fruit bodies of induction.

There have been several studies on the productivity of the Sun mushroom. Figure 17.9 shows some of these investigations; a variation in yield from 0.60 to 2.25 kg of mushrooms harvested in 12 kg of compost is observed.

17.9 Post-Harvest and Marketing

Once the mushroom is picked, excess soil from the base of the stipe must be removed, then fruiting bodies are washed, cut (depending on market demand), and dried (Figure 17.10).

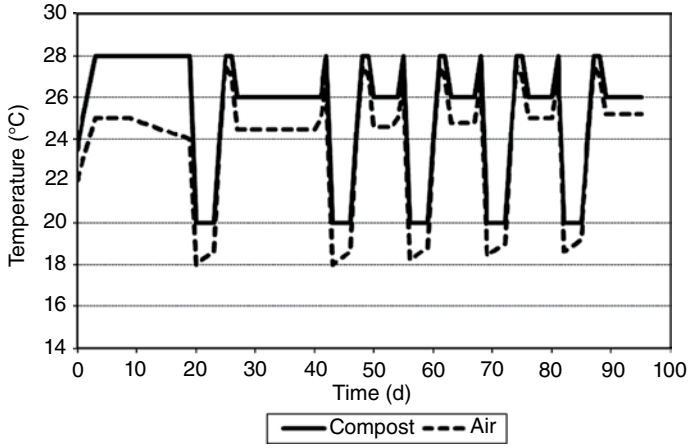


Figure 17.8 Temperatures of growing cycle in the fast cooling method (five flushes).

After washing, some growers divide the fruiting bodies in half and immerse them in a solution with 1% ascorbic acid for 3–5 minutes, and then in a solution containing sodium L-ascorbate 1.5% for 2–3 minutes. Then the fruiting bodies go through a pre-drying with forced ventilation at room temperature to be finally transferred to the dehydrator. The aim of this practice is to maintain the light color of fruiting bodies after dehydration, thus avoiding oxidation (Figure 17.11).

Sun mushrooms are marketed fresh (for culinary use), dried (when harvested with closed cap), or ground powder form (capsule or tea bags). When they are harvested with open pilae due to excess mushrooms in a flush, the grower gets time to wash them, or the pilae open in the dehydrator (as reflected in Figure 17.12, day 06/02; 7:46), the mushrooms are sold at a price 70% below the value of marketed mushrooms with a closed cap.

As Sun mushrooms can be sold whole or ground, it is suggested that the fruiting bodies collected on day 05/02 at 9:34 to 13:37, are marketed whole, in accordance with international standards of the importing country, while fruiting bodies collected after 06/02 day at 7:46, can be marketed crushed, as they have the open pileus to maintain their medicinal properties and their color (Eira, 2003).

The biggest importer of Sun mushrooms is Japan, whose classification standards are as follows:

- Extra mushroom: height 5–8 cm, base width of the stipe of 3.5–5.0 cm, yellow color (clear), and pileus closed.
- Mushroom A: height 3–5 cm, base width of the stipe of 1.5–3.5 cm, yellow color (clear), and pileus closed.
- Fungus B: height of 2–5 cm, width of the foot base of 0.3–3.0 cm, yellow-red color (darkened due to oxidation and dehydration incorrect), and pileus closed.
- Open mushrooms: height of 2–8 cm, width of the foot base of 0.3–5.0 cm, yellow-red color (darkened or not, because the oxidation process and dehydration), and pileus open.

In order to compare the price of fresh Sun mushrooms with the other most cultivated species in the world, the following values in different countries are presented (Table 17.6). In this example the lowest market price in Brazil of $130 per kilo of dried mushrooms (harvested at the optimum point of maturity) was taken, which is around $13 per kilogram of fresh mushrooms, because 1 kg of fresh mushrooms after dehydration represents 100 g of dried mushrooms.

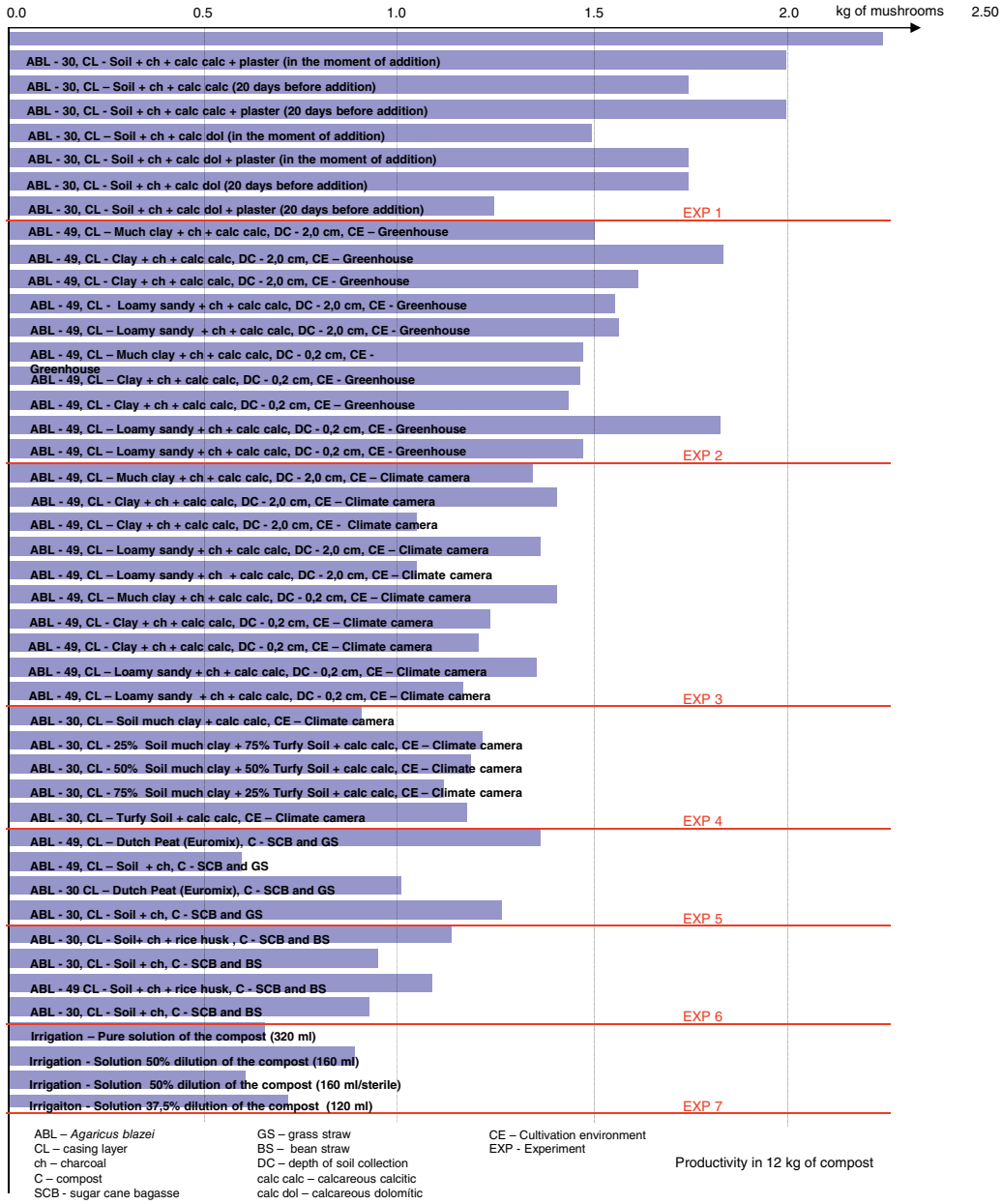

0.0
0.5
1.0
1.5
2.0
kg of mushrooms
2.50
ABL - 30, CL - Soil + ch + calc calc + plaster (in the moment of addition)
ABL - 30, CL – Soil + ch + calc calc (20 days before addition)
ABL - 30, CL - Soil + ch + calc calc + plaster (20 days before addition)
ABL - 30, CL – Soil + ch + calc dol (in the moment of addition)
ABL - 30, CL - Soil + ch + calc dol + plaster (in the moment of addition)
ABL - 30, CL - Soil + ch + calc dol (20 days before addition)
ABL - 30, CL - Soil + ch + calc dol + plaster (20 days before addition)
EXP 1
ABL - 49, CL – Much clay + ch + calc calc, DC - 2,0 cm, CE – Greenhouse
ABL - 49, CL - Clay + ch + calc calc, DC - 2,0 cm, CE – Greenhouse
ABL - 49, CL - Clay + ch + calc calc, DC - 2,0 cm, CE - Greenhouse
ABL - 49, CL - Loamy sandy + ch + calc calc, DC - 2,0 cm, CE - Greenhouse
ABL - 49, CL – Loamy sandy + ch + calc calc, DC - 2,0 cm, CE - Greenhouse
ABL - 49, CL – Much clay + ch + calc calc, DC - 0,2 cm, CE - Greenhouse
ABL - 49, CL – Clay + ch + calc calc, DC - 0,2 cm, CE - Greenhouse
ABL - 49, CL - Clay + ch + calc calc, DC - 0,2 cm, CE – Greenhouse
ABL - 49, CL – Loamy sandy + ch + calc calc, DC - 0,2 cm, CE - Greenhouse
ABL - 49, CL – Loamy sandy + ch + calc calc, DC - 0,2 cm, CE - Greenhouse
EXP 2
ABL - 49, CL – Much clay + ch + calc calc, DC - 2,0 cm, CE – Climate camera
ABL - 49, CL - Clay + ch + calc calc, DC - 2,0 cm, CE – Climate camera
ABL - 49, CL – Clay + ch + calc calc, DC - 2,0 cm, CE - Climate camera
ABL - 49, CL – Loamy sandy + ch + calc calc, DC - 2,0 cm, CE – Climate camera
ABL - 49, CL – Loamy sandy + ch + calc calc, DC - 2,0 cm, CE – Climate camera
ABL - 49, CL – Much clay + ch + calc calc, DC - 0,2 cm, CE – Climate camera
ABL - 49, CL - Clay + ch + calc calc, DC - 0,2 cm, CE – Climate camera
ABL - 49, CL - Clay + ch + calc calc, DC - 0,2 cm, CE – Climate camera
ABL - 49, CL – Loamy sandy + ch + calc calc, DC - 0,2 cm, CE – Climate camera
ABL - 49, CL – Loamy sandy + ch + calc calc, DC - 0,2 cm, CE – Climate camera
EXP 3
ABL - 30, CL – Soil much clay + calc calc, CE – Climate camera
ABL - 30, CL - 25% Soil much clay + 75% Turfy Soil + calc calc, CE – Climate camera
ABL - 30, CL - 50% Soil much clay + 50% Turfy Soil + calc calc, CE – Climate camera
ABL - 30, CL - 75% Soil much clay + 25% Turfy Soil + calc calc, CE – Climate camera
ABL - 30, CL – Turfy Soil + calc calc, CE – Climate camera
EXP 4
ABL - 49, CL – Dutch Peat (Euromix), C - SCB and GS
ABL - 49, CL – Soil + ch, C - SCB and GS
ABL - 30 CL – Dutch Peat (Euromix), C - SCB and GS
ABL - 30, CL - Soil + ch, C - SCB and GS
EXP 5
ABL - 30, CL - Soil+ ch + rice husk , C - SCB and BS
ABL - 30, CL - Soil + ch, C - SCB and BS
ABL - 49 CL - Soil + ch + rice husk, C - SCB and BS
ABL - 30, CL - Soil + ch, C - SCB and BS
EXP 6
Irrigation – Pure solution of the compost (320 ml)
Irrigation - Solution 50% dilution of the compost (160 ml)
Irrigation - Solution 50% dilution of the compost (160 ml/sterile)
Irrigaiton - Solution 37,5% dilution of the compost (120 ml)
EXP 7
ABL – Agaricus blazei
CL – casing layer
ch – charcoal
C – compost
SCB - sugar cane bagasse
GS – grass straw
BS – bean straw
DC – depth of soil collection
calc calc – calcareous calcitic
calc dol – calcareous dolomitic
CE – Cultivation environment
EXP - Experiment
Productivity in 12 kg of compost

Figure 17.9 Yield of the Sun mushroom depending on the strain, compost, the casing layer, environmental conditions, and type of irrigation (data provided by Zied and colleagues from their experiences during 2005–2009).

17.10 Medicinal and Chemical Characteristics of Mushrooms

Mushrooms are starting to be considered as a functional food and a source of physiologically beneficial and non-invasive medicine (CTICH, 2010). The medicinal properties of Sun mushrooms have been highlighted in several studies recently reviewed by Wisitrassameewong et al.

Figure 17.10 Diagram of process of post-harvest: (a) mushrooms picked; (b) washing; (c) clean mushrooms; (d) cutting; (e) dehydrator; and (f) final product for export.

Figure 17.11 Dehydrated mushrooms, light colored (left) packages containing 2 kg of dehydrated mushrooms ready for export (center); and mushrooms of a darker shade that have not passed the antioxidant treatment (right).

(2012). It has traditionally been used to treat many common diseases such as atherosclerosis, hepatitis, hyperlipidemia, diabetes, dermatitis, and cancer (Firenzouli et al., 2007). Among the beneficial properties that have been reported are reductions in the growth of tumors, immunomodulatory activities, immunostimulatory effects, antimicrobial and antiviral activities, and antiallergic effects (Wisitrassameewong et al., 2012).

Detailed information on bioactive compounds and the medicinal properties of various mushrooms can be found in Chapters 21 and 22 of this book, highlighting that one of the main components of Sun mushrooms is β-glucan, which has great importance in the export of

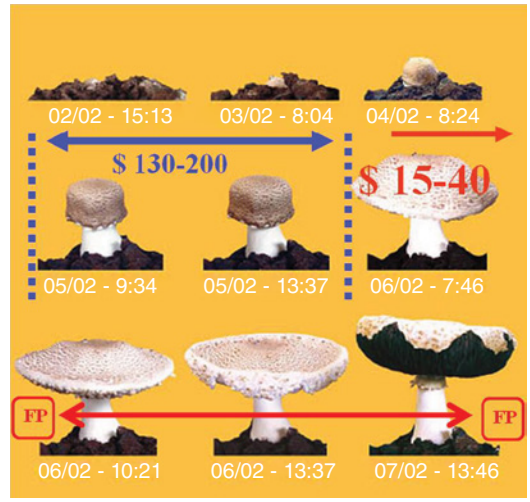


Figure 17.12 States of development of mushrooms and their current market values (dried), * FP–non-standard (crushing of these mushrooms is recommended to give value to the product).

Table 17.6 Price of the most commonly cultivated mushroom species in different countries, comparing them to the commercial price of Sun mushrooms in Brazil.

	Agaricus bisporus	*Lentinula edodes*	*Pleurotus ostreatus*	Sun mushroom
Country/mushroom	**Price of fresh mushroom $ per kg**			
Brazil	~3.0	~5.5	~2.5	~13
USA	~1.2	~3.2	~3.2	—
Mexico	~3.6	~28.0	~2.2	—
Spain	~1.1	~2.5	~1.0	—
Netherlands	~2.6	—	—	—
Ireland	~2.6	—	—	—
China	~2.6	~1.85	~0.92	~13.8
Japan	-	7.0–9.7	3.0–3.7	—
Israel	6.0–8.0	7.0–10.0	6.0-8.0	—
India	2.0–3.0	—	—	

Values reported by the authors of various chapters of this book according to their country of origin. The values for Mexico were provided by Dr. Jose Ernesto Sanchez-Vezquez.

mushrooms. In this regard, Zied et al. (2014) conducted a detailed study based on the influence of cultural practices on the levels of β-glucan in the mushrooms produced, concluding that the greatest contribution to the variation of β-glucan content was strain (35.8%), followed by the casing layer (34.5%), the cultivation environment (15.7%), and the type of compost (9.9%).

Mushrooms are rich in minerals and possess high levels of water, protein, fiber, and carbohydrates; they have low fat content, which make them excellent foods for low caloric diets. Table 17.7 shows some chemical characteristics of Sun mushrooms depending on their physiological state of development (pileus closed and opened) and their morphological parts (stipe and pileus).

It should be noted that the chemical characteristics of dehydrated mushrooms represent important differences when separated by physiological and morphological parts. A clear example is the protein content, which is higher in the pileus compared to the stipe. It may also be noted that a closed stipe has higher protein content than when the mushroom is opened. This influence can also be seen in the content of carbohydrates, energy, and so on. It is noteworthy that the values of cellulose and hemicellulose may represent the values of β-1,4-glucan and branched glucan, as described by Chang and Miles (2004).

There is a trend in value between the chemical characteristics depending on the physiological and morphological parts of the mushroom. For this set of characteristics, the main components retain 74.76% of the variation contained in the original variables (Table 17.7 and Figure 17.13), so there is a tendency for the pileus to be more concentrated with hemicellulose, moisture, and energy; the open stipe has more total carbohydrates. These data can be seen in Figure 17.13.

17.11 Diseases and Pests

Sun mushroom cultivation is susceptible to attack by various diseases (molds, bacteria, and viruses) and pests (flies, mites, and nematodes), mainly due to high temperatures and humidity reached during the production phase (25–29 °C and 82–90%, respectively), ideal for their development.

Disease-causing pathological agents that cause damage directly to mushrooms may be fungal (*Lecanicillium fungicola* and *Mycogone pernicious*) and bacterial (*Pseudomonas tolaasii*), but competing microorganisms (*Trichoderma* spp., *Chaetomium olivacium*, *Papulospora* spp., *Cladosporium* spp., *Diehliomyces microsporus*, *Coprinus* spp.) may also appear, which develop in the compost or the casing layer, reducing the amount of available nutrients and producing toxic substances that retard or prevent the development of mushrooms (Figure 17.14).

The most common pathogenic fungi are: *L. fungicola*, popularly known as "dry bubble" or "Bob", and *M. pernicious* known as "wet bubble"; since ancient times both have been considered as the biggest enemies of mushroom growers because of their aggressiveness and wide geographical distribution (cosmopolitan fungi). They can occur at any stage of development of Sun mushrooms: initial production stage, and final stage of production.

The most common damage is to the formation of tissues (globosa mushroom-shaped and pyramidal; Figure 17.14a). In the initial phase of production, the parasite can invade the tissues of the stipe, causing some openings, leaving the mushroom twisted. The final phase of development of this disease coincides with the end of the mushroom crop, appearing especially in low-tech production environments. The mushroom has some cinnamon colored blotches on the surface of the pileus, resulting from the germination of spores present in the environment (3% loss is common with this type of contamination).

The main sources of fungal contamination are poorly prepared compost and mycelium "spawn" and a casing layer with reduced quality. The presence of spores near the production facilities can also be a problem, especially in low wind protection (curtains, screens, blankets or filters), excessively high humidity, and temperatures above 26 °C.

Bacterial diseases occur on a smaller scale, but can also be prevalent in growing conditions with high humidity and poor ventilation; the main symptom of bacterial contamination is the appearance of pale spots on the pileus that darken to chocolate-brown tones. The presence of pale yellow water droplets in the pileus and stipe are indicators of bacterial contamination

Table 17.7 Mycochemicals characteristics of mushrooms depending on physiological and morphologic characteristics.

	Dry matter (%)	Moisture (%)	Crude protein (Nx4.38, %)	Crude fat (%)	Total carbohydrates (%)	N-free extract (%)	Crude fiber (%)	Ash (%)	Energy value (kcal/100g d.m.)
Closed pileus	9.94 a	90.06 b	37.02 a	1.45 b	53.04 d	47.67 d	5.37 b	8.48 a	351 c
Closed stipe	10.28 a	89.72 b	24.92 c	1.65 b	66.16 b	60.78 b	5.38 b	7.28 b	362 ab
Open pileus	8.64 b	91.36 a	31.03 b	2.91 a	57.79 c	52.18 c	5.61 ab	8.27 a	360 b
Open stipe	10.33 a	89.67 b	18.18 d	1.42 b	73.74 a	67.68 a	6.06 a	6.66 c	364 a
Mean	9.80	90.20	27.79	1.86	62.68	57.08	5.60	7.67	359

	Crude fiber (%)	ADF (%)	NDF (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Closed pileus	5.37 b	22.05 a	43.22 a	21.17 b	7.00 ab	15.16 a
Closed stipe	5.38 b	12.51 c	28.95 b	16.44 c	7.95 a	4.72 c
Open pileus	5.61 ab	16.50 a	43.16 a	26.66 a	6.92 ab	9.68 b
Open stipe	6.06 a	8.52 d	23.83 c	15.31 c	6.68 b	2.04 d
Mean	5.60	14.90	34.79	19.89	7.14	7.90

ADF: acid detergent fiber; NDF: neutral detergent fiber

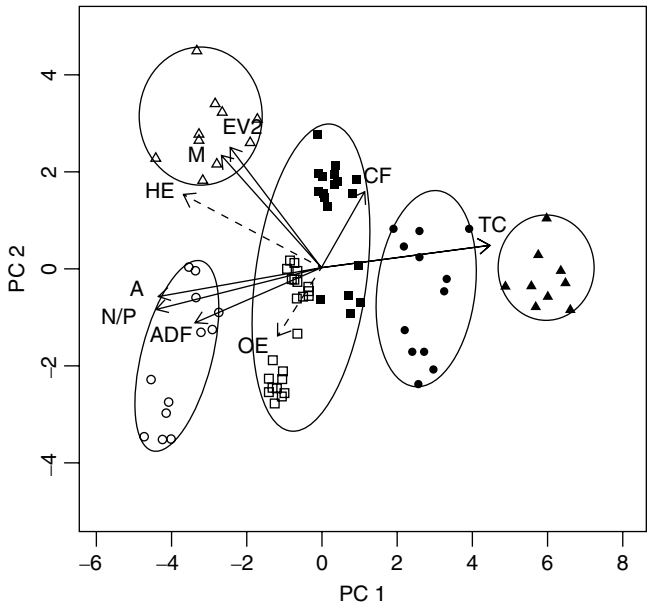
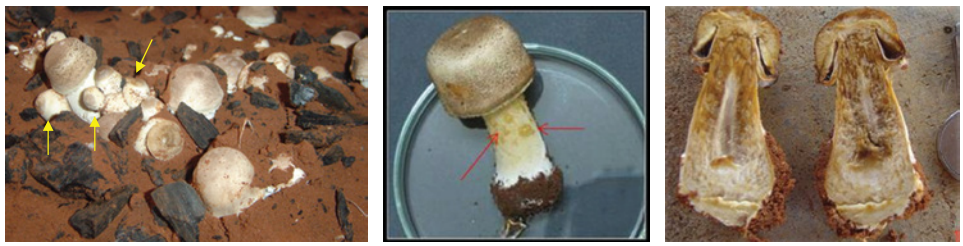


Figure 17.13 Scatter chart scores. Mushroom divided according to their parts and degree of maturation: ○ - Closed/Pileus; ● - Closed/Stipe; △ - Open/Pileus; ▲ - Open/Stipe; □ - Closed mushroom/Pileus + Stipe; ■ - Open mushroom/Pileus + Stipe. EV2 – Energy value; M: Moisture; HE: Hemicellulose; A: Ash; N/P: Nitrogen and protein; ADF: Acid detergent fiber; CE: Cellulose; CF: Crude fiber; TC: Total carbohydrates.



(a) (b) (c)

Figure 17.14 (a) Deformation due to the presence of *L. fungicola* (globose aspect of mushroom), (b) presence of bacteria of the genus *Pseudomonas*, and (c) internal view of mushroom infected by *Mycogone* (wet mass).

(Figure 17.14b). Flies, mites, and other pests contribute to the dissemination of these pathogens.

Between 2004 and 2007 growers in certain regions of Brazil began to observe a reduction in yield due to a pathogen present in their crops, hitherto unknown. The identity of this mycoparasite was revealed in the publication, "First Report of *Syzygites megalocarpus* (*Mucorales*) Web Mold on the Commercial Portabella Button Mushroom *Agaricus bisporus* in North America," by David Beyer in 2013. This provided an explanation of how mushrooms and mycelia in casing were infected (Figure 17.15). Beyer's manuscript confirmed that 60% of the growers lived in Brazil in 2005. Many producers have now left the activity. The problem was correlated with spawn contamination.

Among the pests that attack crops, three deserve greater attention: flies, mites, and nematodes. Flies are attracted by the smell of compost (materials used in the formulation and the composting process), the mushroom and crop residues that end up generating an accumulation of organic matter. Some species of flies move quickly (Phoridae), others are slower (Sciaridae); some during the reproductive process go through the larva and pupa; others such

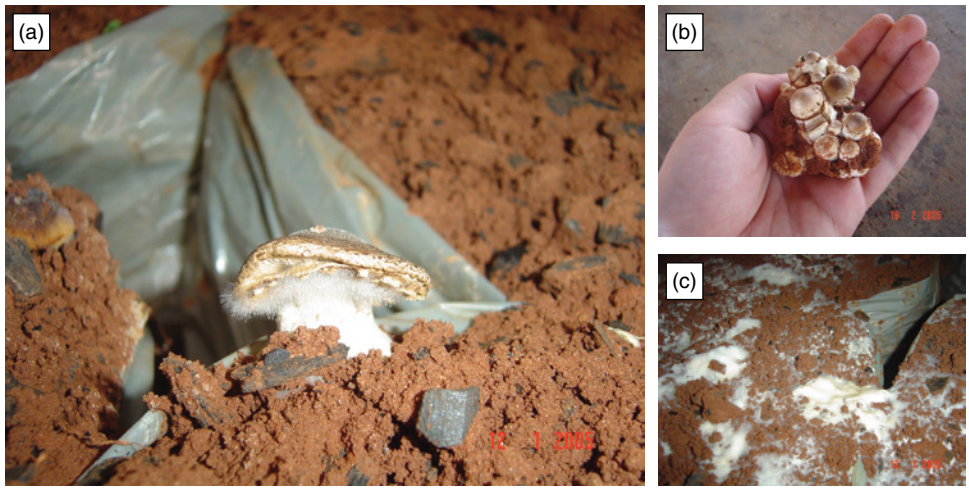


Figure 17.15 (a) Mushroom infected with the mycoparasite *S. megalocarpus*, where it is possible to see its mycelium on lamellae of the Sun mushroom; (b) mushrooms presenting cracks on stipe and brown coloration in the pileus; (c) mycelium of *S. megalocarpus* in the casing layer. (*See color plate section for the color representation of this figure.)*

as the Cecidomidae family reproduce by paedogenesis, that is, several larvae may originate from a single mother.

The life cycle of these flies is completed in 15–42 days; each female may lay an average of 50–200 eggs, half of them females. A recommended method of control to reduce the presence of flies on the premises is the use of light traps (entomological impregnated tail; Figure 17.16), thus preventing their reproduction and egg-laying.

The symptoms are due to larval attack and not the flies, as they make galleries in the pileus and stipe of the mushrooms, depreciating the value of the final product. The larvae feed on the

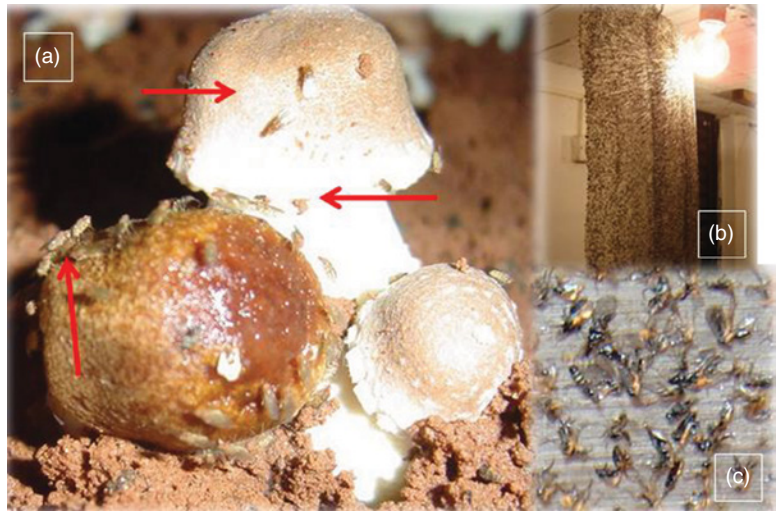


Figure 17.16 (a) Flies in the mushroom, (b) light trap entomological impregnated tail, and (c) close view of the trap with dead flies. (*See color plate section for the color representation of this figure.)*

mycelium of Sun mushrooms and other contaminants. It is also important to note that some flies present at cultivation also feed on organic matter, so great attention should be paid to the stages of production where the mycelium is not present (production compost, pasteurization, conditioning, and development of mycelium).

Mites are commonly found in compost and the casing layer; they have mycophagous habits and can feed on organic matter. Normally they are observed after irrigation of the casing layer, since they have ferruginous coloration and move continuously; the presence of webs in the shelves can be an indicator of the presence of the mites.

Finally, nematodes are microscopic organisms, which have a life cycle of 15–28 days and high fertility (each female lays 30–250 egg). They can be found in the casing layer and in compost, especially with improper pasteurization processes and excess moisture.

It is important to note that there is no biocide (insecticide, fungicide, nematicide, etc.) registered for use in the cultivation of the Sun mushroom, therefore it is very important to adopt cultivation techniques that avoid or minimize the presence of diseases and pests, such as: acquisition of "spawn" quality; preparation, handling, and proper formulation of compost; complete sanitization of cultivation facilities and surroundings; material selection, disinfection, and pH correction of the casing layer; and finally, rigorous environmental control conditions during fruiting and harvesting of mushrooms.

17.12 Spent Mushroom Substrate (SMS)

The SMS of the Sun mushroom has been used in agriculture, forestry (*Eucalyptus* and rubber tree), fruit (citrus generally, strawberry, peach, etc.), and horticulture crops. Several studies have been published on the incorporation of SMS in the production of seedlings of lettuce and tomato and on field crops (Ribas et al., 2009; Marques et al., 2014; Lopes et al., 2015). In the production of tomato seedlings with SMS, seedlings do not present high development in comparison with a traditional substrate used in Brazil; however, after the seedlings were planted in the field the final result was very satisfactory, since the seedlings produced with SMS showed more and better fruit quality (Lopes et al., 2015).

Acknowledgments

We would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – 2015/15306–3 and 2015/24788–1) for financial support.

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18

Cultivation of *Ganoderma lucidum*

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18.1 Introduction

Ganoderma Karst. belongs to the groups Eumycota, Basidiomycotina, Hymenomycetes, Aphyllophorales, Ganodermatacese, and is a genus of wood decaying polypore fungi of economic importance. Up to now, more than 250 *Ganoderma* species have been described worldwide (Wasser, 2011; Kaliyaperumal, 2013). *Ganoderma lucidum* is a species in the genus of *Ganoderma* with numerous pharmacological effects (Zhou et al., 2007b). It is named "Reishi" in Japanese, "Ganoderma" in English, and "Lingzhi" in Chinese. Several species of Lingzhi are permitted for use as the raw materials for human health products (Figure 18.1). In China, Lingzhi is regarded as a herb of longevity and has been used for thousands of years, and Lingzhi strains have been commercially cultivated for the preparation of health products. In the late 1960s, cultivation of Lingzhi had been achieved in China; at that time there were only four *Ganoderma* species both in commercial and research cultivation, which included *G. lucidum* (Leyss. ex. Fr.) Karst., *G. lucidum* (Leyss. ex. Fr.) Karst. var., *G. japonicum* (Fr.) Lloyd., and *G. capense* (Lloyd) Teng. Then, 1-m-long natural logs were used without sterilization for Lingzhi cultivation. A long incubation time (2–3 years) is required to harvest mature fruiting bodies on such substrates. Since the late 1980s, new and improved cultivation methods, short wood log, and substitute cultivation, have been gradually developed and employed for production of Lingzhi. Along with the recognition of medicinal properties of Lingzhi, commercial interests in Lingzhi products sharply increased during 1980–2000, not only in Asian countries but also in North America and Europe (Chang and Buswell, 1999; Chen, 1999). Meanwhile, various different techniques and media have been successfully utilized for Lingzhi production; and developmental products not only based on Lingzhi fruiting bodies, but also based on Lingzhi mycelia or spore powders. Today, almost all Lingzhi growers prefer to adopt short wood-log cultivation or substitute cultivation for production of Lingzhi fruiting body, and the time-saving fermentation techniques (solid-state culture and liquid-state culture) for production of Lingzhi mycelia. With the development of various techniques for culturing Lingzhi, artificial cultivation of *Ganoderma* gradually spread from China, Japan, and the United States to all over the world. This chapter focuses on growing Lingzhi in China for production of fruiting bodies, while fermentation techniques that are preferable in producing a more uniform biomass and metabolites are also summarized. Short wood-log cultivation of Lingzhi is described in detail here mainly based on practices in China. Substitute cultivation is used in enclosing the short logs

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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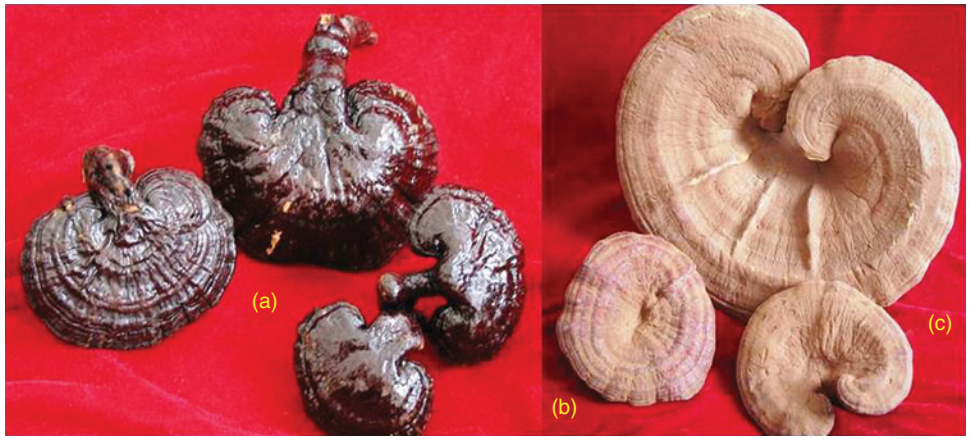

Figure 18.1 Several *Ganoderma* species approved for use in production of health products. (a) *G. sinensis*; (b) *G. tsuage*; (c) *G. lucidum*.

during spawn run (Lin and Zhou, 1998). Genetic breeding and basic facilities coupling with other edible and medicinal mushrooms are also discussed. This chapter is a beneficial reference for shortening the production time and improving fruiting body quality. Some crucial factors and methods of controlling growth and fruiting will be addressed here.

18.2 Growing Conditions of Lingzhi

Lingzhi growth includes several different stages, such as mycelium, primordium, young and mature fruiting bodies, and so on, and each stage has a unique set of requirements in nutritional factors and growth parameters. The nutritional factors such as carbon and nitrogen sources, and growth parameters such as temperature, relative humidity, light, and oxygen supplied at different developmental stages, form the basis for artificial cultivation of Lingzhi.

18.2.1 Nutritional Factors

18.2.1.1 Carbon Sources

A carbon source markedly contribute to the life activity of Lingzhi. It supplies energy for growth and development, composes the fruiting bodies and mycelia, and it is also a component of other substances of *Ganoderma*. The carbon sources are mainly organic, such as sugars, starch, cellulose, hemicellulose, and lignin. The carbon sources commonly used for cultivating the mycelia are glucose and sucrose, while for cultivating fruiting bodies are hardwood sawdust, cottonseed hull, rice straw, wheat straw, corncob powder, and other agricultural by-products.

18.2.1.2 Nitrogen Sources

Nitrogen is one of the major components of protein. Without a nitrogen source, proteins could not be synthesized and then mycelia could not grow. Lingzhi can directly absorb amino acid, urea, ammonia, and other small molecular compounds containing nitrogen. The nitrogen-containing compounds that are usually added to the media are yeast powder and peptone. The nitrogen sources commonly used for cultivation of fruiting body are wheat bran, rice bran, corn powder, ammonium sulfate, urea, and some other compounds containing nitrogen.

The growth and development of Lingzhi require an appropriate ratio of carbon to nitrogen sources. When the carbon-to-nitrogen ratio in the medium is at the range of 15–45:1, Lingzhi mycelium can regularly grow. The optimal carbon-to-nitrogen ratio is 20:1. The growth and sporulation of *G. lucidum* is with the carbon-to-nitrogen ratio of 30–40:1 (Zhou and Deng, 1996).

18.2.1.3 Inorganic Salts

Inorganic elements for Lingzhi growth include potassium, sodium, calcium, magnesium, phosphorus, sulfur, and zinc. Among these elements, the most important ones are phosphorus, potassium, and magnesium (the appropriate concentration is in the range of 100–150 mg/L). Although all elements exist in the substrate, calcium sulfate (gypsum), potassium phosphate monobasic, and magnesium sulfate still need to be added to media, especially calcium sulfate at a dosage of approximately 1%. Calcium sulfate is responsible for adjusting the pH value of the substrate, changing the void fraction of the substrate, increasing ventilation, fixing nitrogen, and increasing the content of calcium and sulfur elements in substrate.

18.2.1.4 Growth Factors

Growth factors are essential substances during the growth and development of Lingzhi, which are involved in the metabolic process of Lingzhi, and mainly include vitamins B_1, B_2, B_6, and biotin. There are little demands for these substances (usually 10 mg/L) during the growth and development process of Lingzhi. Substrates generally contain the growth factors and hence additional substance may be not needed.

18.2.1.5 Moisture Content

Water is an important condition and component for growth. Artificial cultivation of Lingzhi requires a moisture content of the solid-state substrate at approximately 60–65%. Such moisture content can meet the needs of the mycelia growth and will not cause oxygen deficiency symptoms to mycelia. When the substrate is very loose with a larger void between materials, such as when using bagasse as the substrate, the moisture content in the substrate should be increased to 70%.

18.2.2 Environmental Factors

18.2.2.1 Temperature Range

Temperature is a necessary condition for enzymatic reactions during the development process of the mycelia and fruiting bodies. Lingzhi mycelia can grow within a temperature range of 20–35 °C and the optimum temperature is within the range of 25–30 °C. The optimum temperature for the development of fruiting body is within the range of 24–28 °C. When the temperature is below 20 °C, the fruiting body will become yellow and rigid, while it will easily die when the temperature is above 35 °C. During the fruiting body growth and development period, it is not necessary to stimulate with different temperatures. If there is a great difference in temperature, fruiting bodies will readily grow abnormally.

18.2.2.2 Moisture Requirement

During the spawn run, the relative humidity within the incubator (or spawn running room) should be maintained between 60–65%. Moisture control is thus an important strategy for reducing mold growth in the indoor environment. During the fruiting body growth period, the relative humidity within the mushroom house should be increased to a level of 85–95%.

18.2.2.3 Oxygen Level

Oxygen (O_2) is one of the most important elements required to sustain life activities. Lingzhi is a kind of high temperature aerobic fungi. It is necessary to absorb oxygen and release

carbon dioxide during the whole growing period. During development of the fruiting body in Ganodermataceae, if the concentration of carbon dioxide in the cultivation environment is higher than 0.1%, the cap does not grow normally and turns abnormal in shape: an antler-like shape with many branches. Only when carbon dioxide concentration is lower than 0.1% does the fruiting body become large, thick, with a rounded cap, and short stipe.

18.2.2.4 Light Conditions

Lingzhi is very sensitive to light/dark conditions throughout growth. Light inhibits mycelial growth, but weak light can promote the primordium differentiation and pileus formation in the early stage of fruiting body differentiation. Spawn run is usually carried out in the absence of light. Brief exposure to very little light triggers the formation of Lingzhi primordia. In other words, in the absence of light (darkness), individual cells of Lingzhi could not form the primordia and, further, would not easily differentiate into cap cells (pileus). Even if the fruiting body does form, it will grow slowly and develop abnormally. The formation of stipe and cap is also sensitive to light. When the light level is in the intensity range of 20–100 lux, a fully colonized substrate only produces an antler-like fruiting body. At an ambient light level of 300–1000 lux, slender stipes and small caps will be formed, and are strongly curved toward the direction of light. When the lighting level is in the range of 3000–10,000 lux, stipes and caps are normal. Under the optimum lighting level of 15,000–50,000 lux, formation of stipes and caps are optimal (Zhou and Lin, 1999).

18.3 Cultivating Patterns

The cultivation pattern of Lingzhi is the same as that for other cultivated edible mushrooms and can be divided into two major patterns based on whether cultivated on a solid-state medium or in a liquid-state medium. The first pattern involves the production of Lingzhi fruiting bodies, spores, and mycelia biomass, and the second pattern entails the production of Lingzhi mycelia biomass only. Cultivation methods for the commercial production of Lingzhi fruiting bodies and mycelia are summarized as follows.

18.3.1 Production of Fruiting Bodies

The production procedure can be divided into two major stages. The first stage involves the preparation of the fruiting culture, stock culture, mother spawn, and planting spawn, while the second entails the preparation of growth substrates for mushroom cultivation. Currently, the methods most widely adopted for commercial production are the short wood log, and synthetic sawdust bag and bottle procedures (Figure 18.2).

18.3.1.1 Wood-Log Cultivation

Wood-log cultivation technology is defined as using broadleaf tree wood cut to length to artificially cultivate mushrooms. Wood-log cultivation can be divided into three styles: sterilizing a short wood log, wood log, and stump cultivation. Among these styles, sterile short wood-log cultivation has the obvious advantages, such as the shortest growth cycle, high yield, high-quality fruiting body, and many spores (Lin and Li, 2001). Sterile short wood-log cultivation can be described as follows: "inoculation for production of fruiting body after sterilizing the bed-log". Since the 1980s, this method has been used for the production of Lingzhi fruiting bodies in many countries. Its production process can be divided into the following sections: preparation of raw materials (cutting and selection of the wood log), bagging and tying,

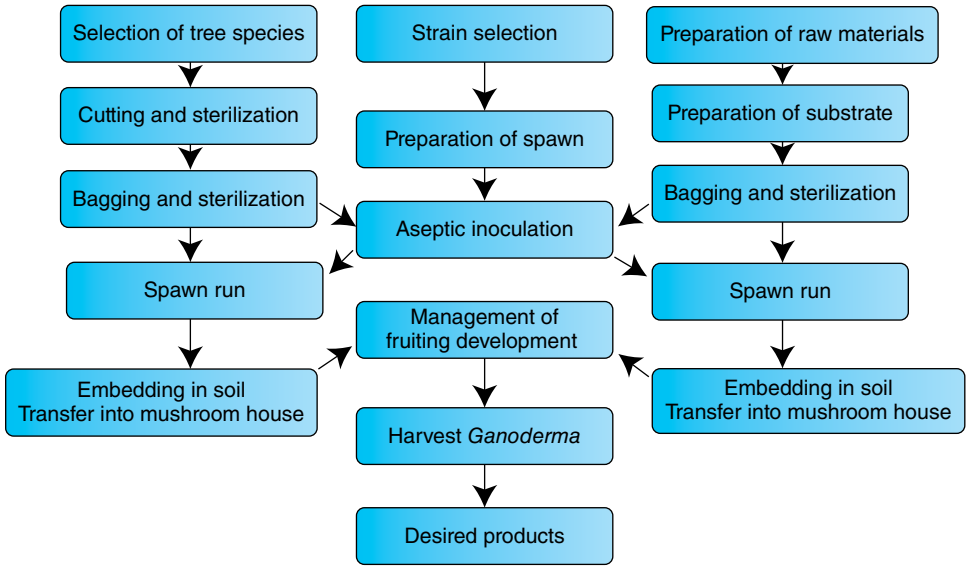

Figure 18.2 Cultivating pattern of Lingzhi for producing fruiting bodies.

sterilization, inoculation, spawns run, embedding in soil (or transfer into mushroom house), management of fruiting body, and harvest.

18.3.1.2 Substitute Cultivation

Substitute cultivation technology is defined as using the sawdust of hardwood, cottonseed husks, or agricultural by-products (offal) to cultivate mushrooms (Triratana et al., 1991). A substrate is a necessary condition for Lingzhi existence. The mycelia growth, fruiting body yield, and various biologically active components in Lingzhi are all influenced by the substrate-related components and culture conditions (Changhai et al., 2013). In the substitute cultivation of Lingzhi, the rich raw materials can be utilized and divided into main ingredients and additives. The main ingredients are traditional cottonseed husk, corncobs, sawdust, and bagasse. The additives include wheat bran, rice bran, corn flour, and soybean powder. In addition, there has been some research on the cultivation of Lingzhi using other forest by-products and industrial residual products as raw materials (Erkel, 2009; Peksen and Yakupoglu, 2009). Substitute cultivation is divided into two types, bottle cultivation and bag cultivation. Comparing the two cultivation types, bag cultivation has more advantages, such as using more substrate, fruiting big bodies, and handy transport, and therefore it is used more widely. Its production procedure includes the following steps: the preparation of raw materials, mixing, bagging (bottling) and sterilization, inoculation, spawn run, embedding in soil (or transfer into mushroom room), management of fruiting development, and harvest. The effects of various kinds of main ingredients and supplements on the yield of *G. lucidum* is different in artificial cultivation (Gurung et al., 2013).

Lingzhi are either gathered from the wild or cultivated on solid media such as wood rather than submerged culture. Many investigators have tried to cultivate Lingzhi on solid-state media for production of fruiting bodies in order to obtain polysaccharides, triterpenoids, proteins (or peptides), and other metabolites used for the production of drugs or health products. However, the cultivation of Lingzhi fruiting bodies is a long-term process that requires between one and several months for the first fruiting bodies to appear, depending on species and substrate

(Kües and Liu, 2000). In addition, the traditional cultivation techniques do not guarantee a standardized product since the composition of the substrate, which affects fruiting body composition, varies from batch to batch (Wagner et al., 2003). Furthermore, Lingzhi fruiting bodies and spore powders have long been used as a folk remedy for promotion of health and longevity but they are actually not suitable for direct use as food or pharmaceutical ingredients because they have a bitter taste and are very hard, which makes it harder for our body to absorb (Qu et al., 2014). With the development of modern biotechnology, fermentation techniques can solve these problems. Since Lin et al. (1973) began to explore submerged fermentation for production of valuable bioactive metabolites (Lin et al., 1973), fermentation techniques have been improving (Wagner et al., 2003; Singhania et al., 2009); there are many investigations focused on Lingzhi mushrooms (Zhou et al., 2012).

18.3.2 Production of Mycelial Biomass

Modern fermentation techniques offer potential to fast produce both mycelial biomass and metabolic products in a shorter time with advantages of reduced space requirements and less chances of contamination (Wagner et al., 2003). High stability and standardization of mycelium grown in fermentation cultures is important not only for producing the desired products, but also might be beneficial for producing Lingzhi-based medicines and nutraceuticals that can achieve higher quality standards and safety (Wasser and Weis, 1999; Elisashvili, 2012). Lingzhi fermentation can be divided into liquid-state fermentation (LSF) and solid-state fermentation (SSF), which are both carried out in bioreactors and involve a series of processes (Figure 18.3) (Zhou et al., 2014). In fermentation, the unitary product cost of SSF is lower than that of LSF. The great advantage of SSF processes is the usage of extremely cheap raw materials as the main substrate. Therefore, SSF is certainly a good way of utilizing nutrient rich solid wastes. Both food and agricultural wastes are produced in huge amounts and since they are rich in carbohydrates and other nutrients, they can serve as a substrate for the production of bulk chemicals and enzymes using the SSF technique (Couto and Sanromán, 2006).

In the fermentation process, the key factors in successfully culturing and gaining the effective products are the level of carbon and nitrogen requirements, pH value, temperature, relative humidity (RH), oxygen, and nutritional components (Habijanič and Berovič, 2000; Malarvizhi

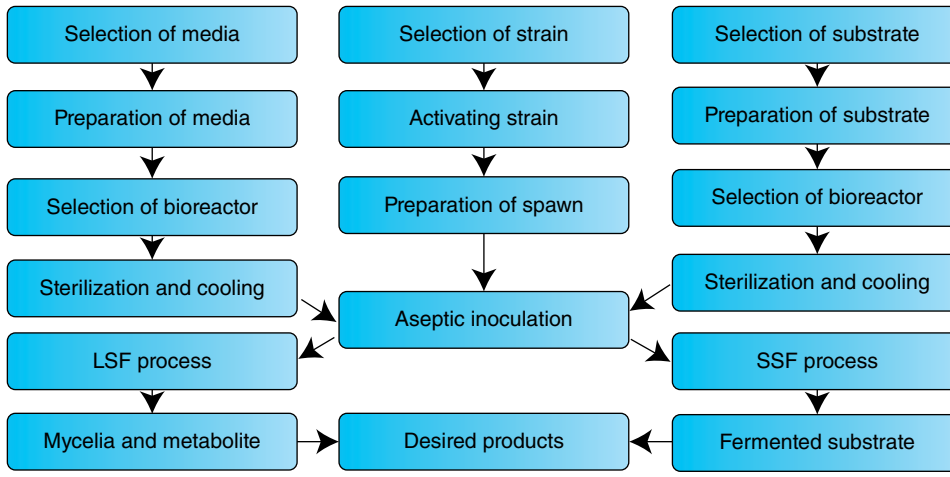

Figure 18.3 Cultivating patterns of Lingzhi for producing mycelial biomass.

et al., 2003). In general, the carbon and nitrogen sources originating from raw materials are different in LSF and SSF, the optimum growth temperature of Lingzhi mycelia differs for various purposes.

18.3.2.1 LSF

LSF, also called "submerged fermentation," is defined as "the growth of microorganisms in liquid-state medium." Both nitrogen and carbohydrates are major nutrient sources in a culture process. In submerged fermentation, carbon sources are widespread, which include the sole carbohydrate source (i.e., glucose, sucrose, fructose, lactose, barley sugar, etc.) and the complex carbohydrate source (i.e., corn flour, soybean meal, distillers' grains, etc.). For nitrogen requirements, an organic nitrogen source is more suitable for the production of Lingzhi mycelial biomass and extracellular polysaccharide compared with inorganic nitrogen sources. In Lingzhi fermentation, there is a lot of information available regarding the influences of various carbon and nitrogen sources (Fang and Zhong, 2002), initial pH value (Fang and Zhong, 2002a), inoculation density control, oxygen supply (Tang and Zhong, 2003), fungal elicitors (Gao et al., 2009), and so on, as well as on the accumulation of ganoderic acid and polysaccharide (Fang and Zhong, 2002b; Tang and Zhong, 2003). In general, LSF methods involve five stages: (1) selection of strain; (2) preparation of culture maintenance medium for different culture phases; (3) inoculation; (4) cultivation of selected strain in Erlenmeyer flasks, seeding tank, and fermenter, respectively; and (5) harvest of Lingzhi mycelia or isolate products.

During the LSF process, it is very important to select and control the appropriate fermentation conditions, such as strain, amount of inoculation, temperature, pH, air flow, and stirring rate. The determination of the indices of the mycelial morphology, concentration, nutrients consumption, and the outward appearance and viscosity of broth could be used as the final quality standard of control fermentation (Chang et al., 2006). Lingzhi fermentation has been done within 25–35 °C, most are done at 30 °C (Wagner et al., 2003). Little information is available about pH effects from studies done in Erlenmeyer flasks since the pH cannot be controlled during the cultivation process. It is possible to study the influence of only the initial pH on growth and metabolite production. The optimum pH value varies for different fermentation purposes. For example, Ling et al. reported that the pH value was related carefully to Lingzhi mycelial growth, intracellular and extracellular ganoderic acids, and exopolysaccharides. The results showed that the optimum pH value was 5.5 for mycelial growth and forming intracellular ganoderic acids, 4.6 for forming exopolysaccharides, and 4.5 for extracellular ganoderic acids (Ling et al., 2009). In addition, if a little magnesium sulfate and potassium dihydrogen phosphate are added into media, the mycelia will grow faster and better.

18.3.2.2 SSF

SSF is defined as "the growth of microorganisms (mainly fungi) on moist solid materials in the absence or near absence of free-flowing water" (Pandey, 2003). The aim of SSF is to bring the cultivated fungi into tight contact with the insoluble substrate and thus to achieve the highest substrate concentrations for fermentation. SSF has several advantages, and is the most appropriate for bioconversion of plant raw materials into value-added products, such as mushroom fruiting bodies, fodder, secondary metabolites, and enzymes. This technology results in several processing advantages of significant potential and ecological importance compared with LSF; in particular, low energy consumption, concentrated nutrient medium, and high volumetric productivity can be achieved in a smaller bioreactor. Moreover, in SSF, concentrated products can be obtained from cheap substrates such as an agro-industrial residue (Singhania et al., 2009). However, the major obstructions for the commercial applications of SSF techniques

have not been completely overcome. They are related to the design and operation of large-scale bioreactors due to problems concerned with the control of parameters such as pH, temperature, aeration and oxygen transfer, moisture, and agitation (Elisashvili, 2012).

Traditional substrates used for Lingzhi SSF include a variety of agricultural products such as rice, wheat, millet barley, grains, beans, corn, and soybeans. However, non-traditional substrates that may also be of interest in industrial development processes include an abundant supply of agricultural, forest, and food-processing wastes, such as wheat bran, soy grits, flakes remaining after extraction of oil, and brewer's spent grain (Hsieh and Yang, 2004; Peksen and Yakupoglu, 2009; Zhu et al., 2010). In general, SSF involves a series of steps such as selection of strains, preparation of seeds, preparation of substrates and sterilization, aseptic inoculation, optimum process parameters, harvesting fermented substrates, and also purification of the end products. There are various important factors that have an immense impact on the process of SSF. These factors include temperature, pH, aeration, water activity and moisture, bed properties, and the nature of solid substrates employed. Among several critical factors, moisture and nature of solid substrates employed are the most important factors affecting SSF process. Selection of moisture depends on the microorganism employed and also the nature of the substrates. Fungi need lower moisture, 40–60% moisture could be sufficient but selection of substrates depends upon several factors mainly related to cost and availability and thus may involve the screening of several agro-industrial residues (Singhania et al., 2009).

In summary, the purposes of both Lingzhi LSF and SSF are to maximize the production of mycelia biomass, or mycelial primary and secondary metabolites such as ganoderic acids or polysaccharides, and so on (Boh et al., 2007), or transfer a chemical compound to another structure-related compound using one or many types of enzyme secreted from fungal cells (Han et al., 2005; Stajic et al., 2010), or produce useful proteins (Murugesan et al., 2007). Therefore, the suitable carbon and nitrogen resources are screened first, the optimum cultivation conditions, including initial pH, rotation speeds (LSF), types of reactor, cultivation temperature should be further confirmed. The fermentation should be done based on the fermentation objectives.

18.4 Production of the Substrate

18.4.1 Preparation of Logs

18.4.1.1 Tree Species and Log Size

Growth of Lingzhi and yield of fruiting bodies are greatly influenced by the tree species (Hou and Liao, 2009). The softwood trees such as the pine, cedar, cypress, and a few hardwood trees such as *Cinnamomum camphora* and *Eucalyptus* spp. can't be used for the cultivation of Lingzhi because of some bactericidal components they contain. Other hardwood trees, such as oak, maple, birch, and locust tree, can be used. Among them, *Castanopsis fargesii*, *C. sclerophylla*, *C. eyrei*, and *C. carlesii* of Fagaceae, *Mountain olive* of Elaeocarpaceae, *Liquidambar* spp. of Hamamelidaceae, and *Mountain peach* of Rosaceae are more suitable tree species for the cultivation of Lingzhi. The diameter of the tree is appropriately 10–13 cm.

The trees are cut down and transported to the site for inoculation. Cut the trees in to 10–13 cm segments and make sure each section is flat. In general, a log with a large diameter should be cut shorter and a log with a small diameter should be longer. The short basswood that is currently popular is about 13 cm in diameter and 12–15 cm long which allows good mycelial running (Chang and Buswell, 1999). The log should be cut to the same length and the section should be flat. The burrs and branches should be cut with a sharp knife to

avoid piercing the plastic bags during the operation process. The freshly cut wood log with high moisture content should be dried for 2–3 days after cutting and tying. When there are 1–2 mm tiny cracks in the center of the section, the moisture content of the log is approximately 35–42% and is suitable for the growth of Lingzhi mycelia. Previous literature reported that the optimum moisture content for the log is about 45--55% (Chang and Buswell, 1999). In winter, the freshly cut wood log can be directly sterilized and inoculated after tying. In spring, the wood log needs to be sufficiently dry to prevent the humidity from influencing the mycelial growth. The wood log can be tied, sterilized, and inoculated after drying for 15 days.

18.4.1.2 Bagging and Sterilization

The wood logs are tied according to diameter, put into plastic bags after ensuring the sections are flat and tight, and then the bags are tied. If the wood logs are too dry, they should be soaked in water for 1–3 h before bagging and sterilization. Then, the plastic bags are sterilized under high pressure or atmospheric sterilization. High pressure steam sterilization is maintained at a pressure of 0.15 MPa (megapascal) for 1.5–2 h. When sterilizing at atmospheric pressure, the plastic bags should be kept in the sterilizer for 10–12 h after the temperature reaches 100 °C.

Sterilization at atmospheric pressure will causes a slight increase of the wood log's moisture content. At the same time, in order to dry the water outside the plastic bags, a gap should be made in the top of the sterilizer after a short time to make the steam overflow quickly, make the air pressure in the pot higher than the outside and reduce cold air into the pot after sterilization. This is also a safe measure to prevent the invasion of mixed microorganisms.

18.4.2 Preparation of Substrates

18.4.2.1 Substrate Formulations

Lingzhi is generally cultivated on hardwood logs or sawdust/woodchips based on formulations. Using plastic bags or bottles as containers for the substitute cultivation, hardwood sawdust is the basic substrate for the cultivation of most mushrooms. Hardwood sawdust, cottonseed hull, crop straw and hull, and bagasse, are usually used for cultivation of Lingzhi. In general, when using sawdust as basic substrate for the cultivation of Lingzhi, the quality of fruiting body is good and the surface of fruiting body is hard. When cottonseed hull is used for the cultivation of Lingzhi, a high yield is available, but poor quality. So, the grower should carefully select raw materials of the substrate formulation that is easy to obtain from the region and suitable for local conditions. Examples of substrate formulations include the following: (1) sawdust 78%, wheat bran 20%, sucrose 1%, gypsum 1%; (2) cottonseed hulls 42%, sawdust 42%, wheat bran 15%, gypsum 1%; (3) cottonseed hulls 89%, wheat bran 10%, gypsum1%; (4) corncob (broken into soybean-sized pellets) 50%, sawdust 35%, wheat bran 15%; (5) corncob 74.5%, corn meal 24.5%, gypsum 0.5%, and plant ash 0.5%. It is noted that these examples are for reference only and can be modified according to the strains selected and the available raw materials in different localities (Chen, 2014; Hossain et al., 2009). In practice, the grower should scale up according to their own needs. The formulations were developed on a laboratory scale first, and have since been successfully duplicated in scale-up operations by mushroom growers all over the world.

18.4.2.2 Packaging Substrate and Sterilization

When preparing the substrates, various ingredients should be accurately weighed and thoroughly mixed for making the synthetic logs in bag or bottle cultivation. Substrates should

be wetted to increase moisture content to approximately 55–65%. The mixture of lime ($CaCO_3$) and gypsum ($CaSO_4 \cdot H_2O$) (2%) in a ratio of 1:1 (w/w) and KH_2PO_4 2% are thoroughly mixed with the substrate. The substrates are poured into heat-resistant polypropylene bags or glass bottles. The bags are plugged with cotton plugs using PVC (polyvinyl chloride) rings, and the bottles are sealed with Kraft paper, then they are autoclaved at 121 °C for 1–2 h. When the bags or bottles are sterilized at atmospheric pressure (at temperatures about 100 °C), the sterilizing time for substrates should be extended to 10–12 h.

18.5 Preparation of Spawn and Inoculation

18.5.1 Preparation of Spawn

Spawn is the seed inoculum used to inoculate the sterilized substrate after cooling, which has two types: solid and liquid spawn. Many growers use solid spawn, while some prefer liquid spawn. The solid spawn is divided into different types based on the substrate used, such as grain spawn, sawdust-bran spawn, dowels, skewers, and grooved woody plug spawn. Pure-culture liquid mycelial spawn can be grown in potato-dextrose broth (PDB) or other formulations (Zhou et al., 2007a).

Preparation of the seed inoculum involves three steps: preparation of stock culture, mother spawn, and planting spawn. Stock culture is usually stored on PDA (potato-dextrose agar) slanted at 4 °C and transferred every 2 months. When preparing mother spawn, cultures are grown on PDA plates at 25 °C for 7–10 d in the absence of light. A 1-cm diameter mycelial plug from the peripheral edge of a 7–10-day-old colony was further used for the production of liquid cultures. The mycelia were incubated in 150 mL PDB medium in a 500 mL flask at 25–30 °C under 150–200 rpm shaking conditions. After 8–10 d, the mycelia are cultivated up to the later log phase to produce an inoculum for liquid culture in fermenter. The appropriate amount of liquid spawn medium is added into the previously sterilized fermenter at 121 °C for 20–30 min and the amount of inoculum produced is 10–15% (v/v). The culture conditions, including cultivation temperature, agitation speed, pressure in fermenter, and aeration rate, are confirmed based on the manufacturer's instruction. When the cultivated mycelia reach the maximum biomass, they should be harvested to use as planting spawn (Wang et al., 2011). Conventional solid spawn is similar to cultivation of other edible mushrooms that are prepared as described (Qiu et al., 2010).

18.5.2 Inoculation

Methods of inoculation are different in liquid and solid cultivation. After sterilization and cooling of the substrate, inoculation should be carried out in a clean and dry place to avoid uninvited contamination. A suitable inoculation volume and seed age should be considered firstly. In wood-log cultivation, an example of inoculation volume is a log with capacity of a square hectometer used as the solid-state spawn of 80–100 bottles. In bag cultivation, localized inoculation methods that involve depositing the spawn on top or on the sides of the substrate block are adopted. Spawn in the bag should be compacted in order to prevent spawn movement. If the bag has residual moisture, the moisture should be poured out before inoculation. Broken bags should be replaced with a new bag or the small hole repaired with tape. Seed age of 30–50 d should be better for cultivation of Lingzhi fruiting bodies (Chen et al., 2013; Hou and Liao, 2009). In LSF, inoculation volume is set up within the range of 5–15% of the liquid medium volume.

18.6 Facilities

18.6.1 General Facilities

General facilities for Lingzhi production include various buildings and equipment, such as an inoculation room, culture room, preservation room, sterilizing room, mushroom house, packing room, waste disposal room and related equipment, laboratory, and other facilities. Among them, the first series of rooms is the primary facilities, which should have the proper ventilation equipment, temperature and moisture management, suitable light-adjustment management, and so on. The roof and walls of the mushroom house should be clean and solid, made of brick or concrete, leaving no gaps to facilitate cleaning and disinfection, except for ventilation windows. Facilities for Lingzhi production are not different from facilities for the cultivation of other mushrooms. The basic equipment mainly includes incubator shaker, fermentation tank, ultra-clean work table, inoculation box, mixer, sack packer, autoclave and stovetop autoclave, and culture shelf.

18.6.2 Equipment System for Preparation of Substrate

The equipment system for preparation of the substrate is a series of mechanical devices for slicing, smashing, mixing, fermenting, sterilizing, bagging, and transferring materials. Substrate production has the characteristics of seasonal intensity. It is a complicated and labor intensive process, so it is completed using some mechanical devices. The slicing machine is applied for cutting branches or wooden strips into thin pieces. It is also used for cutting agricultural straws (e.g., cornstalk and cotton stalk) and then smashing them as substrate. The smashing machine is a simple machine with a small engine for smashing sliced material into pellets for cultivation of Lingzhi. The mixing machine is the uniform mixing equipment for mixing the main ingredients, supplementary ingredients, and water. The bagging machine is bag packaging equipment for placing the mixed material into specially sized plastic bags.

Based on using different materials for the cultivation of Lingzhi, sterilization methods are divided into physical and chemical methods. The physical methods are often used including heat sterilization, fermentation sterilization, and ultraviolet sterilization. Therefore, sterilization equipment mainly includes various sterilizers, such as high temperature and pressure steam sterilizers, normal pressure steam sterilizers, high temperature drying cabinets, and ultraviolet lamps. The equipment system for preparation of strains is a series of special equipments used for isolation and reproduction strains at all grades, which includes inoculation equipments (e.g., inoculation box, clean bench), culture equipments (e.g., cultivation frame, incubators, air-conditioner), and strain preservation equipments (e.g., vacuum freeze dryer, refrigerator). Mechanical devices using for post-harvest handling mainly include dryers, vacuum package machines, and waste bag treatment machines.

18.6.3 Mechanization and Automation Control System

Lingzhi production tends to vary, this includes production of fruiting bodies with industrial intelligent control systems, mechanical facilities, manual modes of production of a gardening style, wood-log production methods, and semi-manual production methods. As a general trend, the Lingzhi industry has changed the courtyard economy mode, using the farmer as a production unit, into an intelligent industrialized production mode and mechanical facilities production model. A variety of production methods coexist all over the world at present. However, all production methods must follow the growth rules of Lingzhi.

In the mechanical production mode, mechanical devices include substrate mixing machine, sawdust bagging machine, air-conditioning, electric fan, and humidifier. A few growers control environmental factors using intelligent control systems, most growers monitor and control major environmental factors based on manual operation methods. Various high pressure steam sterilizers are the main tools for sterilizing various media (or substrates). Many production processes, such as transportation of bags, inoculation, mycelium stimulation, and harvest, mainly rely on manual operation.

In the automatic production model, an intelligent growth environmental control system is usually used to equip a mushroom house and spawn running room. Based on the growth and development rules of edible mushrooms, some environment parameters such as temperature, RH, and CO_2 concentration, are preseted in the computer control system. Using this system, the grower can monitor growing environment parameters of edible mushrooms, calculate exactly the temperature, RH, and CO_2 concentration according to preset target values, and achieve adaptive adjustment of environment parameters by precisely controlling peripheral devices (Lu et al., 2013). Because of the mechanization and automation of production facilities, all production links and adjustment of environment parameters are completed by intelligent devices via computer control. In the process of the preparation of substrate (medium), bagging, inoculation, monitoring spawn running, fruiting body development, and harvest, mechanical devices are used throughout the production process, which include a mixing machine, sawdust bagging machine, inoculation machine, mycelium stimulation machine, and so on. Bag transfer relies on a conveyer belt, and environment parameters are completely adjusted by an intelligent growing environment control system based on different development stages of mushroom. It is noted that an automatic inoculation system, including automatic inoculation machine of liquid spawn and automatic inoculation machine of solid spawn, should be set up in a highly clean room equipped with an air cleaning system (Bian and Liu, 2012).

18.6.4 Other Facilities

A bioreactor, also called the fermentation tank, is a device or container in which certain microorganisms are encouraged to thrive, causing the contents of the tank to ferment and generating a usable substance. With the development of the modern biotechnology, various bioreactors are used for the production of Lingzhi biomass or bioactive metabolites, such as polysaccharides and ganoderic acid (Wagner et al., 2003; Berovič et al., 2012). There are a number of types of bioreactors that are classified into two main groups: suspension and immobilization systems (Zhong, 2010). Stirred tank reactors (STRs) and airlift reactors (ARs) are mainly applied to Lingzhi fermentation (Lee et al., 2007; Habijanič et al., 2013).

STRs are one of the most conventional bioreactors, and are the preferred bioreactors in the research and production of Lingzhi fermentation for biomass or metabolites (Papaspyridi et al., 2011). The stirred tank reactor (Figure 18.4) consists of a tank, mixer, and a series of devices for measurement and control of different parameters in the fermentation process, such as dissolved oxygen concentration, pH, temperature, mixing, and supplementation of all nutrients need to be controlled and optimized. Fundamental design principles and structure of ARs are different from STRs. During the research of Lingzhi LSF, previous studies have been done in volumes of 10 L or less, only a few reported large-scale fermentation in which *G. tsugae* was cultivated in tanks with a volume of 20 L (Wagner et al., 2003; Papaspyridi et al., 2011). Of the research on Lingzhi SSF, the only report available was carried out in a horizontal stirred tank reactor with a total working volume of 30 L (Wagner et al., 2003). Therefore, both LSF and SSF have enormous potential for the production of Lingzhi biomass and metabolites.

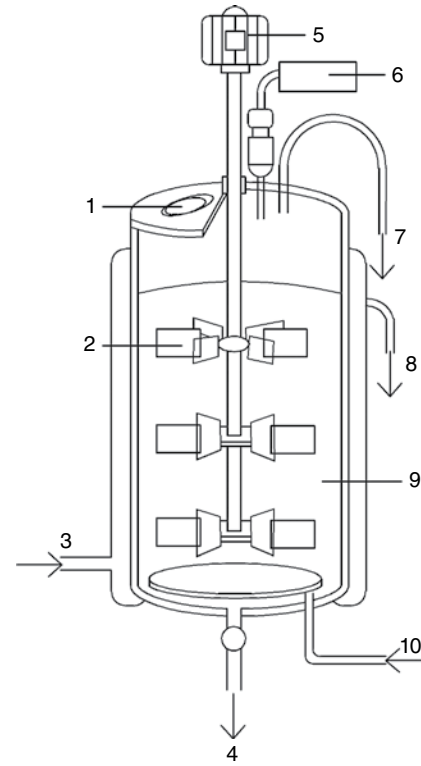

Figure 18.4 Schematic diagram of STRs. 1. Charging hole; 2. Mixer; 3. Cooling water inlet; 4. Clean hole; 5. Electric engine; 6. A device for measurement and control; 7. Blow vent; 8. Cooling water outlet; 9. Liquid-state medium; 10. Sterile air.

18.7 Genetic Breeding

18.7.1 Selective Breeding

Selective breeding (Zhou et al., 2012), including natural and artificial selection processes, is a primitive breeding method that uses artificial means to choose superior strains from nature, for biologically obtaining a new species and reproducing it selectively. The basic methods of artificial selection are tissue isolation and single-spore isolation techniques for obtaining the pure strain, followed by optimization of this strain, and then the required strain is obtained. In fact, during the procedure of mushroom production, the tissue separation method is often applied in obtaining the strain because Lingzhi spores are hard to germinate. A general procedure of artificial selection involves the following steps: tissue isolation → clone screening → strain purification and rejuvenation → cultivation trail of screened strain → selection of superior strain (Chu et al., 2010). Artificial selection is more extensively used in the breeding of animals, plants, and microorganisms, and more commonly used in the breeding of other edible mushrooms than in Lingzhi.

18.7.2 Cross Breeding

Cross breeding technology is the most widely used and effective method in breeding the hybrid strain. The cross breeding of strains has traditionally been accomplished by trial and error, and large numbers of hybrids generated by pairing monosporic cultures; these need to be cultivated to evaluate the production characteristics. The principle of cross breeding is to achieve genetic recombination through haploid mating, and then strains from generation with the parent's

good traits are selected. Cross breeding has been carried out since 1983 in mushrooms with the production of hybrids in *Lentinula*, *Pleurotus*, and *Agaricus*. Subsequently, the technology has been widely used in research on breeding of edible fungi in China and other Asian countries (Chakravarty, 2011). The major goal of cross breeding is to combine desirable characteristics from different strains and create variability in the existing germplasm. That can be obtained only by pairing monosporic cultures. Hybridization for strain improvement of two oyster mushrooms was achieved by a dual culture technique of monospore cultures (Kumara and Edirimanna, 2009). But Lingzhi spores are difficult to germinate under artificial conditions, so the monokaryotic strains which are necessary for the breeding of Lingzhi are not easy to obtain. As a result, the hybridization process of Lingzhi is restricted. Therefore, the protoplast monokaryogenesis technique to obtain the new strain is used in practice (Liu et al., 2014). There have been some reports about artificial cross breeding in which most were selected using protoplast as materials (Dong et al., 2009).

18.7.3 Mutation Breeding

Mutation breeding is a new and more effective method compared to the artificial selection method, which changes genes of the strains and achieves genic recombination. As with other edible mushroom, improving the strains of Lingzhi for better quality and greater yields is the primary breeding goal. Other goals include enhancing the yield of spore powder as well as resistance to high temperature. General procedures of mutation breeding involves the following steps: selection of original strain → preparation of spore (or protoplast) suspension liquid → viable count and mutagenizing → spreading plate for cultivation → picking up desirable strains and inoculation → initial screening → slope culture → re-screening → selection of superior strains. In this breeding method, the selection of the desirable strains from a diverse population generated by natural mutation or induced mutation is one of the key steps. New mutations are usually induced by physical or chemical agents. Mycelia plugs of the actively growing parent strains are subjected to various doses of mutagens and mutated mycelia are assessed in laboratory experiments for the desired selection and are used to prepare mother flasks, spawn, and fruiting bags for production experiments (Chattopadhyay et al., 2014).

Protoplast is mostly used for mutation breeding, and the increased bioactive components, such as polysaccharides, triterpenoids, and organic germanium, are usually regarded as the breeding objectives in Lingzhi breeding. Mutation breeding could not only increase strain mutation rate through simple operation but also provide genetic markers for further cross-breeding and cell fusion breeding. For example, Zhang et al. developed a spore *G. lucidum* strain by using UV inducing mutation technology (Zhang et al., 2014a); a strain with a resistance to high temperature (above 35 °C) was obtained by screening tests under high temperature conditions and the artificial satellite for space mutation treatment (Zhang et al., 2014b). However, it also has some disadvantages. For example, the mutation generation is random and the work selecting the mutant is complicated.

18.7.4 Protoplast Fusion for Breeding

Protoplast fusion is a breeding technique for genetic manipulation in fungi (Cha and Yoo, 1997). This method of hybridization has been successfully used to produce heterokaryons between strains that are incompatible by conventional breeding methods. In the fusion of cell protoplasts, after breaking the cell wall, the different genotypes of protoplasts from various organisms are fused with each other, which is induced by an chemical fusion agent (i.e., polyethylene glycol, PEG) or by electrofusion methods followed by the reversion of the protoplast by plating on osmotically stabilized agar media for regeneration of the cell wall. In that case, the

fusion of isolated protoplast could make the cell genomes from different genera mix effectively, which produces a whole set of genetic exchanges and restructures to generate a new individual. A common method of cell fusion engineering breeding involves the following procedures: selecting parent strain → ensuring genetic markers of parent strain → isolating protoplast from parent strain → regenerating and culturing protoplast → fusing protoplast → regenerating and culturing the fusion → detecting and selecting fusion. In the 1970s, this method had been widely applied in Basidiomycetes breeding (Ferenczy et al., 1974). At the beginning of the 1980s, it was used in edible mushroom breeding in some Asian countries. With the development of techniques for the isolation and preparation of Lingzhi protoplast, protoplast fusion has been gradually applied in breeding the new strains by fusing intrageneric protoplast (Park et al., 1988) and intergeneric protoplast (Bok et al., 1994; Yoo et al., 2002). For example, a protoplast fusion experiment between a cultivar *G. tsugae* Murrill and a field isolate of *G. lucidum* (W. Curt.: Fr.) Lloyd was successfully conducted to obtain an artificial fertile hybrid for enhancing the high and low temperature tolerance and favoring production throughout the year (Chiu et al., 2005). However, protoplast fusion technology has had a major impact on two recent advances in the methodology of fungal genetics: in the mid-1970s through fusion techniques and throughout the decades through their use in transformation-mediated recombination.

18.7.5 Gene Engineering Breeding

Genetic engineering is a set of techniques from molecular biology (such as recombinant DNA) by which the genetic material of plants, animals, microorganisms, cells, and other biological units are altered in ways or with results that could not be obtained by methods of natural mating and reproduction or natural recombination (Sun et al., 2001). The application of recombinant DNA techniques in mushroom research has created numerous possibilities and opportunities for the improvement of yield and quality of mushroom. Using this technology for creating transgenic mushrooms, a DNA sequence from unrelated sources is isolated and then transferred to the commercial strains. Meanwhile, the good characters of the donor strain can be expressed in the host strain, which will become a high-production and good quality strain. The first such transformation was made in *Agaricus bisporus* in 1993 (Mooibroek et al., 1995) and later on applied in other cases also. The general procedure of genetic engineering breeding involves the following steps: selecting donor strains → separating gene → gene reconstruction *in vitro* → transfer the gene into the recipient cell → reproduction and expression of recombinant DNA → selection of new individual. In the process, gene transformation is the key step. Up to now, six kinds of transformation methods have been applied for filamentous fungi, which include protoplast-mediated transformation (PMT), *Agrobacterium tumefaciens*-mediated transformation (Zhang et al., 2014c) (ATMT), electron transfer, biolistic transformation, restriction enzyme-mediated integration (REMI) and lithium acetate. Most of these methods have successfully worked in breeding of Lingzhi (Sun et al., 2001; Kim et al., 2004; Shi et al., 2012).

With the genetic improvement and metabolic engineering of Lingzhi, genetic engineering breeding of Lingzhi has been the subject of further research. Increasing bioactive components, such as polysaccharides, triterpenoids, and organic germanium, became the primary breeding goal. For example, Yu et al. overexpressed the truncated 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) gene that encodes the catalytic domain of HMGR in *G. lucidum* by the PMT method, which increased the transcription of the HMGR gene and enhanced ganoderic acid accumulation (Yu et al., 2014). Zhang et al. structured an over-expression vector carrying a rice OsUgp2 (UDP-glucose pyrophosphorylase) gene, and introduced it into *G. sinensis* via the ATMT method. The results showed that all of the biomass, intracellular, and extracellular polysaccharides were evidently increased in *G. sinensis* (Zhang et al., 2011). In brief, gene

engineering techniques could contribute to new vigor for using secondary engineering to breed a new species of Lingzhi. However, there are a lot of problems existing in edible (or medicinal) mushroom transformation, which include the interference of false positive colonies, the lower integration rate of foreign DNA, the lower expression rate after foreign DNA integration, and the transformant instability. Among these problems, major factors are the lower integration rate and the lower expression rate of foreign DNA.

18.8 Duration, Number of Flushes

18.8.1 Spawn Running

After inoculation, the incubated bags are displayed in the glyph arrangement, stacked in three layers (Figure 18.5), and then set up for spawn running at 26±1 °C in darkness. The RH is adjusted to 60–70% during spawn running. The key management for the next 15 d is mainly ventilation, lowering humidity, and preventing the invasion of mixed microorganisms. When the temperature is higher than 22 °C, the mycelium will germinate within 2–3 d and the hyphae connect into pieces within 1 week.

With the massive growth of mycelia, some droplets appear in the incubated bags because of mycelial respiration. At this stage, the key management is to enhance ventilation and lower humidity. If the incubated bags have too much water, they can be directly pricked with sterile needle to create a pinhole. If the pinhole is relatively large, a piece of gummed paper tape might be pasted outside to keep the halves together. If the temperature is lower, spawn running will be lengthened. At the later stage of spawn running, the bags should be set up in the presence of weak light (about 300 lx). At this stage, weak light will benefit the primordial differentiation. The stronger the light is, the slower the growth speed of Lingzhi mycelia is. It is worth noting that the cloned bags should not be placed in total darkness, otherwise it will result in the

Figure 18.5 Display mode of the incubated bags for substitute cultivation.

formation of undifferentiated primordia (unformed bud). Brief exposure to very little light triggers Lingzhi primordia initiation. Oxygen is also conducive to primordia formation. In contrast, spawn run is carried out in darkness, and less oxygen is required. Primordia of Lingzhi are usually formed within 60–70 days after spawning in log cultivation. Therefore, culture processes of Lingzhi mycelia must go through light training so that Lingzhi primordia initiation is successfully triggered.

18.8.2 Buried Colonized Logs in Soil

The colonized logs are embedded directly in the soil after formation of fruiting body primordia, while leaving the primordia above ground level. Choosing a sunny day, the planting site should be plowed deeply before burying the colonized logs. Turn the soil to a depth of 20 cm; expose the soil for 2 d under the summer sun, then make the mushroom bed. The mushroom bed is 150–180 cm in width, and the length of the mushroom bed varies with the planting plot. Soil with good drainage, such as sandy soil or containing high humus, should be used.

The following is an example: irrigate with water thoroughly before making the mushroom bed, set out the logs alternately in the bed, leave 5–8 cm spaces between each log, while covering with 3–4 cm of thick humus soil or sandy soil. In general, bury 9 logs per square meter. In the early stage of fruiting body growth, high humus soil moisture should be controlled at about 20–25%, while in sandy soil moisture should be about 16–18%. In the late stage, soil moisture of the two kinds of soils should be controlled at 18% and 15% respectively in order to prevent the growth of mold (Chen et al., 2013). In addition, the planting site should be a light loam, which is aerated better and more water permeable; the depth of the buried soil is appropriate for covering 2 cm. Burying at a greater depth creates poor ventilation, affecting fruiting body germination, which causes some stipe and soil mixing, and loss of nutritional value. A small depth is of no benefit for retaining moisture and temperature. Alternatively, soil-buried log cultivation of *Ganoderma* species can also be carried out in the open air in the wild.

18.8.3 Management of Fruiting Body Development

The development process of fruiting bodies can be artificially divided into different stages (Figure 18.6). The most crucial management practice during pileus differentiation is to increase ventilation to reduce CO_2 concentration, along with high humidity and diffused dim light (Chen, 2002). The question arises whether to use sawdust synthetic log cultivation or natural log cultivation, when the mycelium has colonized into the substrate completely, the bags are transferred to a fruiting room at 25 ± 1 °C, 80–90% RH to develop the primordia to stalk, then to promote cap formation. Nowadays almost all growers prefer to adopt the soil-buried log cultivation indoors in order to produce high-quality Lingzhi products. In general, after the colonized logs are embedded in soil or transferred to a fruiting room, the fruiting body of Lingzhi could grow out within 7–14 days if temperatures continue to be higher than 25 °C. The bud comes up from the soil or substrate with a white top and brown base (Figure 18.6.a). When the stipe reaches a certain length, given appropriate conditions, such as ventilation, temperature, humidity, and illumination, it will differentiate the pileus (Figure 18.6.b). Among these conditions, the temperature is the key factor. The temperature for fruiting body formation is in the range of 25–35 °C. The fruiting body of the best quality has a dense texture and a deep brown color cortex at 25 °C. The fruiting body grows faster, but of poor quality at 28 °C. The fruiting body cannot be differentiated under conditions of continuous high temperatures (above 35 °C) or continuous low temperatures (below 18 °C). The management strategies during pileus differentiation involve some measures, such as water spray, aeration, light, preventing diseases and insects, harvest, and drying.

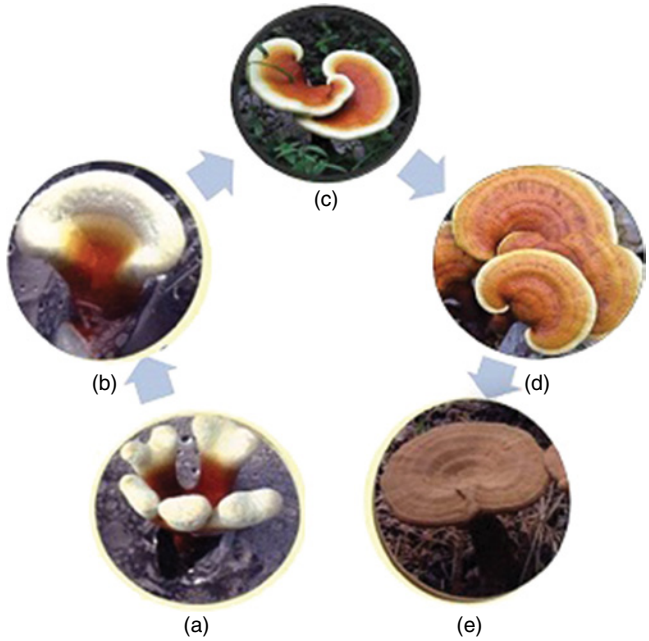

Figure 18.6 Schematic diagram of development process of Lingzhi fruiting body. (a) Bud-breaking stage; (b) Bud-developing stage; (c) Developing stage; (d) Growth stage; (e) Maturity stage. *Source:* Zhou, 2012. Reproduced with permission of Springer. (*See color plate section for the color representation of this figure.)*

It should be noted that Lingzhi is a fungus whose fruiting body primordial differentiation is conducted in the constant-temperature condition. Variable temperature is not beneficial to the differentiation and development of fruiting body. When the temperature dramatically changes, it's easy to generate a ring of differentiation with uneven thickness and abnormal pileus, and it will result in a bad commodity. The sarcomatous primordium can be formed at 12–14 °C, the stipe primordium can be formed at 15 °C as the lowest temperature and the pileus can be formed at 22 °C as the critical minimum temperature. The development phase of pileus (Figure 18.6.c) will take 25 d when the average temperature is 18 °C, and take 18 d when then average temperature is 25 °C. Therefore, the management of Lingzhi production should focus on the regulation of moisture, ventilation, and lighting. In order to improve the level of the commodity, a regular inspection is necessary after the Lingzhi fruiting bodies grow out, for example, leave one thick individual only on each line, cut the branch appearing on the stipe with a knife. When the caps become completely red and the white margins disappear (Figure 18.6.d,e), fruiting bodies can be harvested. Harvest by cutting the stipe (stalk). Keep only 2 cm of the stipe with the pileus.

After harvesting, fruiting bodies should be air dried under the sun or with heat (60 °C), which will take 2–3 days. When drying, the fruiting bodies should be placed with the underside of the pileus facing down. During cloudy or rainy days, the fruiting bodies should be dried at low temperature (60 °C). Improper prolonged drying lowers the quality of the product; the underside pore surface turns dark brown or becomes contaminated by molds (Chen, 2002).

18.8.4 Number of Flushes

Flushes refers to the harvesting time or number of harvests. If so desired, continue cultivation under the optimal growth parameters for second and third flushes, although the subsequent flushes have lower yield, especially the third flush. When harvesting the first flush, adding water (nutrition solution) to the substrates for spawn running should be paid more attention. After the 2-week period of spawn running, second flushes could be harvested. In substitute

cultivation, Lingzhi could be harvested for two flushes. From primordia formation to fruiting body for harvest takes approximately 25–30 days. If an appropriate criterion is used for optimizing management, 1 kg of dry substrate can produce 100–120 g of dry fruiting body of Lingzhi. In practice, the reasons for increasing harvesting time are (1) utilizing the unused substrate in the bottom of the bags and the accumulation of nutrients of residual mycelia, (2) promoting the mycelial metabolism by adding nutritional supplements, (3) promoting the transfer and accumulation of nutrients by adding water, and (4) promoting primordial differentiation and being convenient for absorbing nutrition solutions (Zheng et al., 1995).

18.9 Diseases and Pests in the Cultivation of Lingzhi

18.9.1 Main Lingzhi Diseases and Insect Pests

18.9.1.1 Fungi and Harm Caused

As a result of being infected by pathogens or influenced by poor environmental factors including temperature, humidity, and toxic chemicals in the artificial cultivation and production, *G. lucidum* will develop metabolic disorders and abnormal changes in the morphological and physiological features, thereby reducing the yield of Lingzhi. This phenomenon is defined as Lingzhi disease. Here, pathogens causing fungal diseases and their damage are summarized as follows (Wang, 2009).

Trichoderma spp., a class of pathogenic fungi, is responsible for causing Lingzhi mildew during its cultivation process. The disease is characterized by rapid onset and serious harm. In the fruiting body development stage, mycetes grow in the back of the Lingzhi cap after fungal invasion. When mucus is exuded from the cap surface of Lingzhi, the fruiting bodies stop growing and become green and mildewy, causing fruiting body deterioration, and loss of commercial value. In the mycelial growth stage, *Trichoderma* spp. could cause the mycelia to lose culture substrates after the fungal invasion, while fungal mycelia also secrete toxins, inhibiting the mycelial growth, and then scrap the cloned bags.

Mycogone pernicioso is responsible for causing Lingzhi brown rot disease during the cultivation process. When Lingzhi is infected by this fungus, the fruiting body grows abnormally showing an enlarged stipe and a small cap, while the surface of the fruiting body is covered with white fluffy mycelia, which turns brown and produces brown droplets.

Neurospora is a genus of Ascomycete fungi that often causes Lingzhi diseases in the mycelial growth stage. When *Neurospora spp.* infect the cloned wood log (or the cloned bags), the surface of the log (or bag) grows loose mesh hyphae, and the rapid growth of the fungi seriously affects the mycelial growth of Lingzhi. *Aspergillus* spp. is also a class of invasion fungi in Lingzhi cultivation, and often causes Lingzhi mycelia growth to slow down or stop by the invasion of polluted soft substrate. These fungi often affect Lingzhi production at the fruiting body development stage. In the early period, sticky mesh mycelia may appear on the surface of the covered soil, the fruiting bodies in the soil may stop growing, or show symptoms of diseases. These diseases will seriously affect the yield and quality of Lingzhi.

18.9.1.2 Insect Pests and Harm Caused

Some pests are often found to damage the mycelium or fruiting body during cultivation, which could directly result in reduction of output of commercial Lingzhi. Meanwhile, bite wounds are vulnerable to bacterial infection, causing concurrent diseases, resulting in greater losses. Therefore, this is a very important aspect of prevention and treatment of the pests. Linhzhi pests are mainly insects belonging to the genera Lepidoptera, Coleoptera, Isoptera, and Collembola,

such as snout moths, beetles, termites, and nitidulid beetles, and other small animals such as snail cochlea, nematodes, and slugs. Pests in the cultivation of Lingzhi vary in different cultivation locations. Frequently occurring pests mainly include larvae of moths from the Liodidae, Geometridae, Hypogastruridae, and Termitidae families, also some well-known insect species, such as *Anemapogon gerasimovi*, *Diomeacremate butler*, *Drosophila melanogaster*, *Dolichocybe perniciosa*, and *Limaxflavus linnaeus* (Wang, 2009).

Larvae and adults of most insects mainly damage the Lingzhi fruiting body. In general, early instar larvae feed on the cap surface and edges. After 3 days, the larvae eat directly into the fruiting bodies inside, while discharging large amounts of waste. Termites, both *Odontotermes termites* and *Macrotermes barueyi,* mainly eat the cloned wood log, to compete for nutrition with Lingzhi mycelia, influencing mycelial growth and development. Larvae of *Drosophila melanogaster* feed on mycelia and substrates in substitute cultivation, which often cause water-soaked rot on the surface of the cloned bags. When larva bore into the fruiting body, they cause atrophy and decay. *Dolichocybe perniciosa* not only feeds on Lingzhi fruiting bodies and mycelia, but also spreads mycetes (such as *Trichoderma* spp., *Neurospora* spp., and *Fusarium* spp., etc.), which result in the invasion of mixed microorganisms in cultivation of spawn and influence spawning and cultivating of Lingzhi.

18.9.2 Prevention Strategies for Diseases and Pests

Diseases and pests of Lingzhi are closely related with the selection, environmental sanitation, soil disinfection, pest control, and management of the Lingzhi cultivation site. During the process of substitute cultivation, the main reasons for the occurrences of diseases are incomplete sterilization of the substrate, pollution in the inoculation process or cultivation process, breakage of sawdust cultivation bags, and impure spawn (Zhou et al., 1999). The improper selection of cultivation site, thatch, straw, and mat for shade in the greenhouse, and the covered grass of the Lingzhi bed are the main reasons for insect damage.

Prevention and treatment of diseases and pests should adhere to the principle that prevention is more important than treatment. The cultivation environment should be firstly improved in order to reduce and eliminate the occurrence of diseases and pests. Comprehensive prevention and treatment strategies in combination with ecological control and chemical control should be established to reduce the harm level of diseases and pests, and to ensure cultivation of *G. lucidum* with a high yield and good quality. Suggested strategies are presented as follows.

1) Agricultural control. *G. lucidum* species with strong resistance to diseases and pests, good quality, and high yield should be selected to make sure that spawn doesn't introduce pests, such as viruses, bacteria, and mites. The substrate should be completely sterilized by high (or normal) pressure. Environmental hygiene of the Lingzhi cultivation site should be of a high standard. Remove equipment polluted by bacteria, and carry out disinfection and deinsectization of the cultivation site.
2) Physical control. The cultivation site should be managed to avoid being a breeding ground for insect eggs and fungal spores. A cultivation site used for years can't be used again and again, and growers need to rotate beds to prevent diseases and pests and provide soil nutrient sources for Lingzhi growth. If possible, it's better to use a fly net and sunshade net for isolating the culture chamber and Lingzhi greenhouse, preventing pest invasion. Using UV and ozone for disinfecting the inoculation apparatus and cultivation site should be considered.
3) Chemical control. The chemical agent is the most effective measure to prevent diseases and pests in the cultivation of Lingzhi. However, when chemicals are used improperly, it's easy to damage human health. Chemical control is considered only when necessary. Chemical

agents produced by local manufacturers are various and should be selected according to manufacturer instructions. For example, beta-cyfluthrin 4.3% or fluorine cyanide 1000 times is often used for the prevention of insect pests, such as tineoid, noctuid, termite, and limaxcan. Chlorothalonil coupled with thiram 30% or prochloraz 40% is often used for spraying in mildew disease prevention, Alternaria alternata, brown rot, and so on.

18.10 Medicinal Values

Traditional medicines have held an important status in health care systems in developing countries, and Lingzhi has been one of the most prescribed traditional medicines. A great deal of work has been carried out on the therapeutic potential of Lingzhi. Approximately 400 different bioactive compounds have been isolated and identified from the fruiting bodies, spores, and mycelia of Lingzhi, which mainly include polysaccharides, triterpenoids, nucleotides, sterols, steroids, fatty acids, proteins/peptides, and trace elements. It has been reported that these components have a number of pharmacological effects such as immunomodulation, antitumor, sleep promoting, antibacterial, antiviral (including anti-HIV), anti-aging, and anti-ulcer properties (Sanodiya et al., 2009). Some active components are used as the raw materials of health products that have been verified by many scientific reports. Among these ingredients, triterpenes, and polysaccharides have attracted considerable attention as they have been shown to possess diverse and potentially significant pharmacological activities. Except for a few molecular mechanisms of their pharmacological functions, most are not completely clear (Zhou et al., 2007b). With the exception of polysaccharides and triterpene/triterpenoid compounds, fungal immunomodulatory protein (FIP) is also an important bioactive component with immune regulating activity, which is the most promising active ingredient developed by modern biotechnologies (Li et al., 2011a).

18.10.1 Polysaccharides

Ganoderma polysaccharides (Ganopoly) were the first discovered ingredients, and have been investigated extensively for many years. α or β-(1–3)-, (1–6)-glucans and heterosaccharides with different combinations of glucose, mannose, galactose, xylose, fucose, as well as arabinose, have been extracted from different species, and molecular weight ranges from thousands to millions of Dalton (Nie et al., 2013). Up to now, more than 200 polysaccharides have been isolated from the fruiting bodies (Chen et al., 2008), spores (Bao et al., 2002), mycelia, and cultivation broth of Lingzhi. They have shown various important bioactivities, such as immunomodulatory, antitumor, antioxidant, hepatoprotective, anti-hypertensive, and so on. Ganopoly are glucans, β-1–3 and β-1–6 D-glucans, mainly consisting of neutral polysaccharides of glucose units; about one third of the polysaccharides consist of (1 → 3)-β-D-glucan containing β-(1 → 6)-D-glucosyl branches. Among them, most glucans with antitumor activity contain a branched glucan core with (1 → 3)-β-, (1 → 4)-β- and/or (1 → 6)-β-linkages and have an average molecular weight of 1,050 kDa (Zhou et al., 2007b). Both *in vitro* and *in vivo* studies suggest that the antitumor activities of Ganopoly are mediated by its immunomodulatory, anti-angiogenic, and cytotoxic effects. Ganopoly affects immune cells and immune-related cells including B lymphocytes, T lymphocytes, dendritic cells, macrophages, and natural killer cells (Xu et al., 2011). In addition, recent data also suggests that Ganopoly suppresses tumorigenesis or inhibits tumor growth through direct cytotoxic effect and anti-angiogenic actions (Weng et al., 2009; Sun et al., 2011). However, widely accepted antitumor effects of Ganopoly arise from the enhancement of the body's immune system rather than direct cytocidal effects. (Yuen and Gohel, 2005).

18.10.2 Triterpenes

Triterpenes/triterpenoids are one type of the most important biologically active components in Lingzhi. Groups of triterpenes have received considerable attention because of their well-known pharmacological activities. Up to now, more than 150 triterpenoids (mostly lanostane-type triterpenes) have been reported from the fruiting bodies, spores, mycelia, and culture media of Lingzhi (Ma et al., 2011). These triterpenes could be divided into the C30, C27, and C24 compounds according to the number of carbon atoms and based on the structure and the functional groups. In general, triterpenoids have molecular weights ranging from 400–600 kDa and their chemical structures are more complex than the group of lanostanes because of their highly oxidized state (Huie and Di, 2004; Cheng et al., 2010). Triterpenes possess significant bioactivities, such as antioxidation, hepatoprotection, anti-allergy, anti-hypertension, cholesterol reduction, as well as inhibiting platelet aggregation, due to the inhibition of enzymes such as β-galactosidase, angiotension converting enzymes, cholesterol synthase, and so on (Huie and Di, 2004). For example, Lin et al. (2003) reported that a triterpene fraction could inhibit the growth of human hepatoma cells via suppressing protein kinase C and activating mitogen-activated protein kinases and C2-phase cell cycle arrest (Lin et al., 2003); Zhu et al. found that the triterpene fraction exhibited the highest effect by testing the ingredients, against pyrogallol induced oxidation on erythrocyte membrane and Fe (II)-ascorbic acid induced lipid peroxidation in liver mitochondria (Zhu et al., 1999). Some literature also reports that a number of triterpenes show significant anti-HIV-1 protease activity (Zhu et al., 1999; El Dine et al., 2008).

18.10.3 Fungal Immunomodulatory Proteins

FIP, initially isolated from *G. lucidum*, is a small molecule protein with similar structure and immunoregulatory activity to phytohemagglutinin and immunoglobulins. It is a well-documented bioactive component after the polysaccharide and triterpene compounds. Since Kino and colleagues first isolated FIP from *G. lucidum* mycelia, another 10 FIPs: ling zhi-8 (LZ-8/FIP-glu) and FIP-LZ9, FIP-gts, FIP-fve, FIP-vvo, FIP-gja, FIP-gmi, FIP-gsi (Zhou et al., 2009), FIP-tvc (Li et al., 2011b), and FIP-nha, have been isolated and identified from *G. lucidum*, *G. tsugae*, *Flammulina velutipes*, *Volvariella volvacea*, *G. japonicum*, *G. microsporum*, *G. sinensis*, *Trametes versicolor* (Li et al., 2012), and *Nectria haematococca* (Bastiaan-Net et al., 2013), respectively. FIPs, with a molecular weight approximately 13 kD, is composed of 110--114 amino acids, and is poor in His, Cys, and Met but rich in Asp and Val. The peptide is acetylated at the N-terminus. FIPs have immunomodulatory functions that play an important role in antitumor, anti-allergy, stimulation activity on immune cells to produce a variety of cytokines, anti-transplant rejection, and so on, which indicates a promising application of FIPs for medicinal care (Zhou et al., 2005; Li et al., 2011a). Among the various functions, the antitumor effects arouse particular interest. For example, FIP-gts expressed in *E. coli* could effectively control the metastasis of human lung adenocarcinoma A549 cells, so it had efficient antitumor effect (Liao et al., 2008). Cong et al. recently found that FIP-SN15, a recombinant DNA sequence generated by DNA shuffling technology between FIP-glu and FIP-gsi (Wang et al., 2013), and FIP-glu could cause growth arrest as well as apoptosis in human glioblastoma U-251 MG cells *in vitro* (Cong et al., 2014). In short, numerous studies have proved that FIPs had antitumor activity both *in vivo* and *in vitro*, it would become a potential candidate for developing antitumor drugs with high efficiency, low toxicity, high specificity, and conducting cancer chemical prophylaxes and antitumor therapies.

Today, Lingzhi is still widely accepted as a valuable health supplement and herbal medicine worldwide for the prevention and treatment of a variety of ailments, and has been well

recognized for its high nutritional and medicinal value. Traditional medicine prefers to use Lingzhi in integrative medicine, for example in conjunction with chemotherapy or radiation therapy in treating cancer patients, and in recovery to a level that enables them to undergo surgery for late-stage cancer patients. It has been documented that Lingzhi produces a huge number of bioactive compounds. The 431 secondary metabolites are isolated from various *Ganoderma* species. Among them, over 380 terpenoids (ganoderic/lucidenic acids, meroterpenoids) and 30 steroids are isolated from Lingzhi, these isolates not only stimulate the immune system but also modulate specific cellular responses by interfering in particular transduction pathways (Baby et al., 2015). Currently, Lingzhi produces beneficial effects not only as drugs but also as a novel class of products with different names: Dietary supplements, functional foods, nutriceuticals, mycopharmaceuticals, which provide health benefits through everyday use. Several types of Lingzhi products are available on the market today. These products mainly include (1) artificially cultivated fruiting body powders, hot water or alcohol extracts of these fruiting bodies; (2) biomass or extracts from mycelium harvested from submerged liquid culture grown in a fermentation tank or bioreactor; (3) naturally grown, dried mushroom fruiting bodies in the form of capsules or tablets; and (4) spores and their extracts. There is no doubt that Lingzhi-based products can serve as superior dietary supplement products. Unfortunately, standardization of various Lingzhi-based products is still in its early stages all over the world (Wasser, 2014). We must set up a series of internationally recognized standards and protocols for the production and testing of Lingzhi-based products.

Acknowledgments

The author would like to thank Professor Hong Ji (Changshu Institute of Technology, Jiangsu Province, China) for his help to photograph the Lingzhi artificial cultivation samples. I thank my graduate students for their help in proofreading and editing the English of the final manuscript.

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19

Naturally Occurring Strains of Edible Mushrooms: A Source to Improve the Mushroom Industry

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Mushroom cultivation worldwide is based mainly on three species: *Agaricus bisporus* (both the white button mushroom and the brownish Portobello), *Pleurotus ostreatus*, and *Lentinula edodes*. Mainly, Southeast Asian countries have a food culture that values mushroom diversity, so they appreciate and consume many species of mushrooms.

Although 2500 species of mushrooms have been reported as edible, only 50 species are produced on substrates. From these, only 25 are commercially produced and about eight are the most frequently produced around the world (Chang, 1993).

The current production of mushrooms is based on the domestication process of many strains that were collected in the wild over the last 50 years, and were selected because of their quality and productivity.

One of the problems in mushroom production is that commercial strains sometimes reduce their production performance after several consecutive subcultures or after a long period of storage in culture medium, leading to a reduction in the yield.

Biological efficiencies can sometimes be raised by optimization of cultural conditions, such as combining different substrates or adding nutritional supplements. Nevertheless, these practices are not always successful in recovering the production of a commercial strain's performance. An alternative solution could be to search for new naturally occurring strains for cultivation, which could result in finding highly productive or quality strains.

If we take into consideration the increasing world population, the challenge today is to find new species and new strains that could help to improve mushroom production for the forthcoming years.

19.1 Edible Species and Their Cultivation

Species can be classified based on how they obtain their nutrients (Largent et al., 1986): (1) symbiotic, growing in association with other organisms; (2) pathogenic or parasitic, causing harm to other organisms; and (3) saprobic, growing on dead organic matter.

The symbiotic species of edible mushrooms are associated with trees and the production of fruiting bodies requires high investment and time. For this process, young seedlings roots have to be inoculated under microbiological controlled conditions with the fungi, then moved to greenhouses in plant pots and finally planted on adequate areas (Morte et al., 2008), so the

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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mycorrhized trees can grow together with the fungi. An example of this is truffle production, for which 6–10 years are needed to obtain the first yield (Lefevre and Hall, 2001).

Parasitic fungi cause great economic losses in agriculture so they are mostly eliminated. Huitlacoche is an example of a parasitic fungi (*Ustilago maydis*) that is cultivated on corn plants in Mexico for human consumption (Valverde et al., 1995). On the other hand, saprobic species can be cultivated with widely well-known techniques for mushroom production (Stamets, 2000). The different methodologies applied for mushroom cultivation try to produce a suitable substrate where mushrooms can fruit. Within the group of saprophytic mushrooms, there are two very different sub-groups: (1) mushrooms that grow on the ground are defined as *humicolous* and (2) mushrooms that grow on wood and are defined as *xilophagous*. The first subgroup, which could be represented by *Agaricus* spp., requires mostly a very complex substrate known as compost, which is very selective. The second subgroup, which could be represented by *Pleurotus* spp. or *Lentinula edodes*, does not require a composting process and can fruit on many sterilized or pasteurized lignocellulosic substrates (Albertó, 2008). Humicolous species are not easy to produce in culture, probably because of the composition and quality of the soil where they naturally grow. Abiotic factors like mineral composition, organic matter, soil structure, and so on, could influence mushroom growth. Probably, biotic factors like bacteria or fungi could also help to induce primordial formation in soil. These very selective types of soils (substrate) are very difficult to obtain on a mushroom farm. On the other hand, most xilophagous species produce fruiting bodies easily. These species have a wide battery of hydrolytic enzymes that are inducible by substrate, and degrade it to releases nutrients for growth and fruiting (Mata and Savoie, 1998).

There are many wild edible mushrooms all around the world that are mostly known and consumed by "mycophagous" people in each region. It is interesting to use this "popular knowledge" and search for these "new" naturally occurring species that could be intensively produced in mushrooms farms, increasing the range of new mushroom products.

19.2 Steps for the Domestication of Naturally Occurring Species

There are many steps that have to be carried out prior to the intensive production of a naturally occurring species of mushroom. Strains are obtained from nature, from protected areas, forests, or simply where they grow, frequently on the grass/lawns near houses.

Steps for domestication of naturally occurring species of mushroom can be summarized as follows:

1) *Strain isolation*: the first step is to obtain the strain (isolation). The strain can be obtained from a small piece of flesh from the context of the pileus or the stem which is cultured on a rich agar medium. An expert mycologist has to certify that the isolation obtained belongs to the specimen collected and discard any contamination. It is also very important to be absolutely certain that the mushroom collected is an edible species. If there is any doubt about this subject the strain should be discarded. Another option is to make a test of edibility using mice or rats.
2) *Determination of optimal conditions for mycelia growth*. The objective of this step is to obtain the highest growth in the shortest period of time. Thus, parameters such as pH, temperature, nutrients, and so on, should be determined. These optimal conditions should be used for spawn production and incubation period or any stage for vegetative growth of mycelia.
3) *Spawn production*. Spawn can be produced by inoculating the strain selected on sterilized cereal grains (Sinden, 1932). The type of grain (sorghum, wheat, oats, etc.) and humidity of the grains, are also critical to optimize the time required for spawn production.

4) *Selection of substrates*. Different types of substrates should be tested to determine which most productive are. If we are working on xilophagous mushrooms it is advisable to test the substrates where mushrooms grow in nature. It is also useful to try the substrates which are available and abundant in the area where the species would be cultivated. Firstly, it is suitable to assay substrates formulated with 100% of one type of component (e.g., 100% of *Poplar* sawdust) and then it would be appropriate to assay mixtures of different components.
5) *Incubation period*. At this point the mycelia run has to be evaluated for each substrate. Time required for the total colonization of mycelia has to be determined. Type and size of containers (bags, boxes, trays, etc.) should be evaluated since they can influence growth and time of colonization.
6) *Light requirements for primordia formation*. For some species light is necessary for primordia formation and for some others primordia can normally grow in the absence of light, for others primordia do not need light for their formation but they do for normal development into fruiting bodies. Recently, Colavolpe and Albertó (2014) proposed a simple model to determine the light necessary for a mushroom to produce primordia based on the use of a fishbowl divided into equal compartments. One of these and the upper part were coated with opaque black polyethylene plastic film to prevent light from coming in and to keep it completely dark, the other compartment was covered with a transparent polyethylene plastic film. Light quality can also be studied (Kurtzman and Martínez-Carrera, 2013).
7) *Induction conditions*. Some environmental factors are known to have an influence on triggering off the induction mechanisms for fruit body production. These are temperature, soaking, and air exchange increase. Generally, a drop of 10 °C in room temperature induces primordia formation in most cultivated mushrooms. Some species such as Shiitake or *Polyporus teniculus* (= *Favolus tenuiculus*) have a better fruiting response when they are submerged in cold water (Omarini et al., 2009).
8) *Use of casing soil*. Some humicolous species require a layer of soil or peat for fruiting. The casing soil modifies the relation between O_2 and CO_2 on the substrate surface, providing water and a physical support for the growing mushrooms. As a consequence, the use of casing soil, the use of different components, and the thickness of the layer have to be studied in detail (Kalberer, 1985).
9) *Supplements selection*. The supplements are nutrients mainly rich in carbohydrates or protein that help to increase yields. They should preferably be of low cost and easily accessible. There are many supplements that are commercially produced for the production of *Agaricus bisporus*, additionally other supplements such as wheat bran, sunflower hull, waste malt from the brewery industry, soybean flour, and so on, can also be assayed.
10) *Production conditions.* There are many parameters that should be studied for mushroom production, these are: optimal temperature for fruiting, air exchange, watering (volumes and type of watering) light requirements (type of light, photoperiod), and air exchange (CO_2 tolerance, speed of air exchange, etc.).
11) *Crop parameters*. Many crop parameters should be defined, these are: biological efficiency (BE%), productivity, number of flushes, resting time, percentage of yield by flush, and so on (Salmones et al., 2005; Uhart et al., 2008).
12) *Mushroom parameters*. These are the morphological characteristics of the species such as diameter of pileus, length of stem, color, smell, and so on.
13) *Post-harvest*. This is to determine how to handle, cool, clean, sort, and pack the species. It is also important to determine shelf life and how to extend it (Hammond and Royston, 1975; Brennan et al., 2000).

14) *Nutritional composition*. As it is the contribution of a new species and a new food, nutritional composition should be determined. Protein, carbohydrates, fat, fiber, and ash content should be defined. It is also useful to determine which amino acids, minerals, and vitamins the new species could provide.

Taking into account that all these steps are complex, effort and time-consuming, before the domestication of any species it is advisable first to determine if the species can fruit using the *A. bisporus* technique for saprobic mushrooms, or the *P. ostreatus* technique for xilophagous mushrooms. Many naturally occurring species can be isolated, it is possible to produce spawn, and they easily colonize the substrate, but only a few will produce fruiting bodies. The reason why this happens is still unknown. There are abiotic and biotic factors that could influence this process. Among the abiotic factors are the composition of the substrate, minerals, and so on; environmental variations produce changes in humidity, temperature, oxygen, and so on. The biotic factors are very difficult to study and are related to the vast biodiversity of organisms present in the soil, which could influence mushroom fruiting. Those organisms could help to promote and trigger the fruiting mechanism in mushrooms.

19.3 Finding New Species for the Mushroom Production Industry: A Look Back at the Last Few Years

In recent years, many studies have been published showing the methodologies to obtain fruiting bodies of some naturally occurring species. Mounce (1923) managed to obtain fruit bodies of *Coprinus commatus* under controlled conditions. Albertó (1997) obtained fruiting bodies of *Agaricus pseudoargentinus* using *A. bisporus* compost. In this work, six naturally occurring species from the environs of Buenos Aires (Argentina), *A. bisporus*, *A. campestris*, *A. fiardii*, *A. nivescens*, *A. pampeanus*, and *A. pseudoargentinus* were cultured under growing conditions similar to those required by *A. bisporus*. All strains grew well on sterile wheat grains, allowing optimum spawn production (Albertó, 1997). Colonization of compost took between 11 and 18 days after spawning. Only *A. bisporus* and *A. pseudoargentinus* produced fruit bodies. *A. pseudoargentinus* produced pinheads after 42 days of casing and developed fruit bodies at 18 °C but with low yield (Figure 19.1), reaching 230 g/10 kg of compost (Albertó, 1995). Recently, Salmones et al. (2012) studied the naturally occurring species of *A. bisporus* in Mexico; the biological efficiencies (BE) obtained ranged from 40.3 to 95%. Navarro and

Figure 19.1 Production of *Agaricus pseudoargentinus* in *A. bisporus* compost.

Savoie (2015) selected wild strains of *Agaricus bisporus* and produced high yields of mushrooms at 25 °C.

Ruegger et al. (2001) produced fruiting bodies of *Oudemansiella canarii* in a period of 60 days. The largest basidiomata production was obtained in the compost with sugar-cane bagasse with a BE of 55.66%.

Lechner and Albertó (2000) studied *Lentinus tigrinus,* a species with a fleshy pileus, strong odor, and agreeable taste. In order to determine the optimal conditions for the production of this species, three substrates based on *Salix* sp. sawdust, wheat straw, and supplements were tested in 500 g dry weight bags at two different fruiting temperatures. Naturally occurring strains of this species were incubated at 30 °C. Primordium initiation could be observed 11–16 days after induction conditions began. This species produced highest yields with a BE of 62% with supplemented sawdust at 25 °C (Figure 19.2). When bags were reduced to 100 g dry weight, spawning run time was reduced from 28 to 30 and 10 to 14 days, and BE increased more than 100% (Lechner and Albertó, 2007).

Hu and Chu (2008) determined the conditions for the cultivation of the edible mushroom *Hypsizygus marmoreus* with a BE of 33%. Borges Da Silveira and Wright (2002) studied *Polyporus tenuiculus*, a naturally occurring species from Central and South America that is consumed by different ethnic groups in the region. To determine the optimal conditions for fruiting body production, Omarini et al. (2009), assayed two strains on wheat straw and

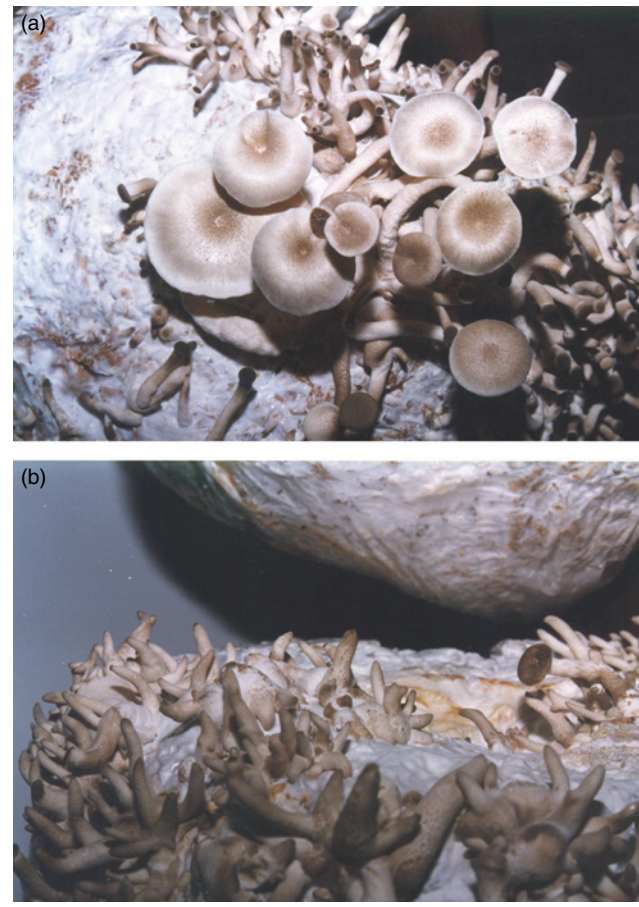

Figure 19.2 Production of *Lentinus tigrinus* on sawdust. Detail of pileus (a) and massive primordia formation (b).

Figure 19.3 Production of *Polyporus tenuiculus* in bags. (*See color plate section for the color representation of this figure.)*

sawdust with and without supplements. Sixty days of incubation at 25 °C were needed to produce a solid block. The highest yield was obtained with supplemented willow sawdust. In a second experiment, different supplements were used to improve the BE and to determine the quality traits and its biodegradation capacity. The highest yields (Figure 19.3) were obtained on sawdust with 25% of supplements reaching 82.7% of BE. *P. tenuiculus* showed a capacity to degrade sawdust, causing a decrease of 67.2–74.5% in cellulose, 80.4–85.7% in hemicellulose, and 60.6–66.2% in lignin content at the end of the cultivation cycle. This species can also be produced in logs (Figure 19.4) using wood of *Populus* sp. and *Eucalyptus* sp. (Albertó and Omarini, 2012). This is a promising species both for commercial production and for its potential use in the degradation of other biowastes. Further studies were made; sensory analysis was made and compared with *P. ostreatus* (Omarini et al., 2010a). Also, volatile composition and nutritional quality of this species grown on different agro-industrial waste was determined (Omarini et al., 2010b). Recently, Omarini et al. (2015) proposed this species to be used to obtain new aroma compounds with potential applications in food and pharmaceutical industries since it has the ability to transform spent *Eucalyptus* essential oil industry wastes by solid state fermentation.

With the objective of improving yields in *Agrocybe cylindracea* (Brig.) Singer, Uhart et al. (2008) studied naturally occurring and commercial strains of this species. *A. cylindracea* is an excellent edible mushroom appreciated for its culinary properties and pleasant odor (this mushroom is known worldwide as *A. aegerita*, nevertheless we use the name *A. cylindracea* (Uhart and Albertó, 2007) because this is the correct name. A screening of genotypes and substrates to select the most productive strains was made. Twelve commercial and naturally occurring *A. cylindracea* strains from different continents were cultivated on wheat straw in order to compare their BE. Those strains that obtained high yield were tested with different supplements. The Asiatic naturally occurring strain cultivated with soybean flour supplementation achieved an average BE of 179% (Figure 19.5), to our knowledge the highest reported for this species (Uhart et al., 2008).

Figure 19.4 Production of *Polyporus tenuiculus* in logs.

Figure 19.5 Production of *Agrocybe cylindracea* in supplemented wheat straw. (*See color plate section for the color representation of this figure.)*

Lechner and Albertó (2011) studied the yield values and morphological variations of fruiting bodies obtained from the cultivation of 14 *Pleurotus* strains isolated from naturally occurring specimens from Argentina, including *P. albidus*. They were tested on supplemented *Salix* sawdust, wheat straw (W), and supplemented wheat straw (SW). In general, wild strains had a good performance on W or SW. The highest yield was obtained with *P. albidus* on wheat straw with a BE of 171.3% that overcame in 82% the yield obtained for the commercial strain of *P. ostreatus* in the same substrate (Figure 19.6). This species, requiring culture conditions similar to those of *P. ostreatus*, can be an interesting resource for food production in the tropics, since it is found everywhere from Costa Rica to Buenos Aires (Albertó et al., 2002) and is

Figure 19.6 Production of *Pleurotus albidus* on supplemented sawdust.

adapted to warm weather. It was also possible to find a strain of *P. ostreatus* with better BE than the commercial strain assayed. Because of the high yields and the good quality of mushrooms obtained, we proposed *P. albidus* as a new species for intensive industrial cultivation (Lechner and Albertó, 2011).

Manjunathan and Kaviyarasan (2011) produced and studied the nutrient composition in the wild and cultivated edible mushroom *Lentinus tuber-regium* fruiting the mushroom on paddy straw. Recently, Colavolpe and Albertó (2014) determined the optimal conditions needed to cultivate *Gymnopilus pampeanus*, a species belonging to the family Cortinareace. They produced this species on *Populus* and *Eucalyptus* sawdust (Figure 19.7). They found that light was necessary for a normal development of primordia. The highest BE was obtained on *Populus* sawdust reaching a mean of 70.67%.

Harada et al. (2015) produced the Andean-Patagonic edible mushroom *Grifola gargal* using several strains and the commercially available mixed hardwood used for *Lentinula edodes* cultivation.

Figure 19.7 Production of *Gymnopilus pampeanus* in bags. (*See color plate section for the color representation of this figure.)*

Agaricus blazei, the medicinal Brazilian mushroom, is one of the best recent examples of how one naturally occurring species can reach an important economic status within the mushroom production in one country. This species has become an important export product for Brazil, reaching higher prices in comparison to other mushrooms (Souza Dias et al., 2004). This is why many researchers studied how to improve the yields (Andrade et al., 2008; Colauto et al., 2010, 2011; Zied et al., 2010, 2011). This species was found in the state of "Sao Pablo" and was produced mainly for its medicinal properties (Mitzuno, 2002; Firenzuoli 2008). At present, this is one of the "new" species added to the list of cultivated species for industry.

19.4 Conclusions

Naturally occurring species could be a very large and important source of "new" species to be cultivated worldwide, providing high quality food and probably acting as functional foods. Many naturally occurring strains could offer adaptative advantages to be locally produced, such as disease resistance or capacity to be cultivated in extreme conditions of temperature and humidity. The study of naturally occurring species could be a useful practice to improve yields, to introduce *new* species to markets, and to preserve germplasm of fungal species before their natural environments are disrupted or destroyed by human action. These strains will support the future world mushroom production.

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20

Spent Mushroom Substrate Uses

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20.1 Introduction

Many species of mushrooms are cultivated worldwide. Global production increased to about 27 billion kg in 2012 (Royse, 2014). According to Royse (2014), 85% of the world production comes from five genera: *Agaricus bisporus* and *A. subrufrescens*, (30%), *Pleurotus* sp. (27%), *Lentinula edodes* (17%), *Auricularia* sp. (6%), and *Flammulina* (5%).

The production of any and all species results in significant residual material after cultivation. Every kilogram of mushrooms produced results in 5–6 kg of by-product (Ma et al., 2014).

Environmentally conscious management of this coproduct of mushroom cultivation has prompted governments and researchers to address the potential use of these residual materials.

Knowledge of horticultural mushroom production practices is crucial to the research and intended post-crop usage of this coproduct of mushroom production. The *Agaricus* group of commercially produced mushrooms are cultivated typically on a straw or hay base, amended with animal manures and gypsum. The materials initially undergo a two-phase composting process, one at high temperature (up to 85 °C) and another for pasteurization and conditioning (beginning at 60 °C and decreasing to about 45 °C). The colonization stage of this mushroom fungus is followed by covering the surface of the colonized compost (or casing) with a layer of peat, top soil, or other suitable material for fruitification. Within 2.5 weeks, mushrooms are visibly ready for harvest. After about 2–3 weeks of mushroom harvest, the growing material is considered spent. Many mushroom facilities are designed for a post-crop heat treatment to eliminate insect and disease transmission to subsequent or neighboring crops. After which, the spent growing material is removed and the chamber is readied for a new crop. The spent material may be placed in piles where it leaches and continues to compost uncontrolled.

Various *Pleurotus* species are cultivated on wood sawdust and various plant fibers that are amended with locally available proteins and carbohydrates to optimize its growth requirements. The materials are generally not composted as per the *Agaricus* group. The wood sawdust may be aged or the plant fibers hydrated for several days. The growing materials are sterilized, pasteurized, or chemically treated to augment the selectivity of these materials for the oyster mushroom fungus. At the end of several mushroom harvests, the growing material is considered spent. It may be heat-treated before being removed from the growing chamber and piled as per *Agaricus*.

Lentinula edodes is either cultivated on natural logs or on a "synthetic" medium formed in "logs." Natural log production utilizes various species of trees. Trees are cut down after leaf fall,

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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cut into lengths of about 1 m, inoculated shortly after cutting, and incubated for about 1 year before fruitification initiation. Mushrooms are harvested about twice per year for several years, depending on climate and log diameter. Once production ceases, these logs are considered spent. Shiitake as well as *Auricularia* sp. and *Flammulina* sp. are also produced on sawdust, straw, corn cobs, or mixtures thereof. Starch-based additives from cereals are often added to optimize the nutritional needs of the fungus. The growing materials are generally sterilized. After several harvests, the growing material is considered spent. As with *Agaricus* and *Pleurotus*, the spent materials may be heat-treated before removal from the growing facility. They, too, are piled where leaching and composting occurs.

This chapter will outline the possible utilities of spent mushroom substrate (or spent mushroom compost) as reflected in published research. One of the first events that formally addressed the research and uses of spent mushroom substrate was through a symposium held in Philadelphia, Pennsylvania in 1994 (American Mushroom Institute, 1995). This author (Rinker, 2002) summarized the varied uses of spent mushroom substrate for a conference held in Cuernavaca, Mexico and in book chapters published by *MushWorld* (Rinker et al., 2004; Rinker, 2005). Since these articles, the number of research articles addressing spent mushroom substrate usage has increased substantially. Phan and Sabaratnam (2012) have provided an excellent review for the potential uses of spent mushroom substrate with emphasis on enzymes for bioremediation, animal feed, and energy feedstock. And Paul Staments (2005) in *Mycelium Running: How Mushrooms Can Help Save the World* details the many practical applications for fungi in solving today's environmental challenges.

The purpose of this chapter is to provide a spectrum of the research investigations on the use of spent mushroom substrate (SMS). At the time of writing, there were over 600 references to the research and uses of SMS from various cultivated mushroom species. The attempt has been to illustrate the bread of the research efforts rather than include every reference to these efforts. Characteristics of spent substrate are outlined, and the following uses for spent substrate are noted: bioremediation, crop production, reuse in the cultivation of mushrooms, food for animals and fish, and pest management.

20.2 Characteristics of Spent Substrate

SMS is a generic term and often is treated as such in the refereed literature by many research authors. However, SMS is by no means generic. Researchers have been quick to research SMS, but regrettably many peer-reviewed articles do not provide a specific reference to mushroom species from which the spent material was derived, a list of materials on which the mushrooms were cultivated, horticultural conditions, post-crop management, age of spent material, its storage time or post-crop composting, or its physical and chemical descriptions. These reflect the characteristics of the SMS used in the research, its repeatability, and the success of its proposed utilization. *Agaricus bisporus* raw materials are similar within regions but still vary such that the characteristics of the spent substrate should be enumerated before usage (Jordan et al., 2008a,b). Mushroom crops, as a precaution against insect and disease transmission to other crops, should be post-crop heat-treated *in situ.* This process is intended to kill unwanted pests, but at the same time it kills the mushroom fungus and affects the microbiology of the substrate (Kleyn and Wetzler, 1981; Ntougias et al., 2004; Raymond et al., 1997). When the substrate is removed from the cultivation room, it may not be used immediately in its application. Thus, it may compost or weather/leach, further affecting the physical and chemical characteristics of the material (Lohr et al., 1984b; Iiyama et al., 1995; Levanon and Danai, 1995; Lemnaire et al., 1985; Szmidt and Chong, 1995; Gerrits, 1997a; Chefetz et al., 2000; Maher et al., 2000; Becher

and Pakuła, 2014; Beyer, 2015). Changes in the SMS as a result of storage need to be taken into account before its intended usage.

Various authors have documented the impact of *A. bisporus* weathering on surface and ground water, covering such topics as: chemistry of leachate (Guo et al., 2001; Guo and Chorover, 2004, 2006), nitrates in ground water beneath sandy terrace soil in intensive vegetable production (Maynard, 1993a,b, 1994a), impact on water quality through applications to agriculture land (Wuest et al., 1991; Wuest, 1992; Wuest and Fahy, 1992; Pannier, 1993; Kaplan et al., 1995), the release of sulfate-sulfur, potassium, calcium, magnesium (Stewart et al., 2000) and inorganic-N (Stewart et al., 1998a), and the effect on adjacent surface water (Reed and Keil, 2000).

The SMS of *A. bisporus* continues to compost when it is stock piled after production, affecting health (Cobb et al., 1995) and producing odors. The odorous components and its management have been addressed by Bazemore et al. (2000), Heinemann et al. (2003), and Velusami et al. (2013).

20.3 Bioremediation

Bioremediation is the use of living organisms such as bacteria, fungi, or green plants, to remove or neutralize unwanted contaminants in air, soil, or water. SMS has been assessed as a partial solution to environmental contaminations.

20.3.1 Air

Agaricus bisporus spent substrate has been evaluated as a mix with other materials for removal of H_2S (Shojaosadati and Siamak, 1999) or volatile organic compounds (Mohseni et al., 1998; Mohseni and Allen, 1999).

20.3.2 Water

Waste water is generated from many and varied industrial activities. These waters may be contaminated with heavy metals from mining operations, dyes used in textile or plastic industries, phthalate esters used in the production of plastics or cosmetics, sulfides from mining or non-mining activities, or pesticides from agricultural operations.

Spent substrate from *Agaricus bisporus* has been evaluated for treatment of metal-contaminated water from coal mines (Dvorak et al., 1992; Stark et al., 1994; Anon, 1997), acid mine drainage (Chang et al., 2000; Newcombe and Brennan, 2010) in wetland environments (Karathanasis and Thompson, 1990; Vile and Wieder, 1993; Wieder, 1993; Stark and Williams, 1994; Stark et al., 1995, 1996; Tarutis and Unz, 1995; Manyin et al., 1997), textile dyes (Toptas et al., 2014), nickel-contaminated mine water (Hammack and Edenborn, 1992), sewage (International Organic Solutions Corp., 1996), waters polluted with radioactive elements and heavy metals (Groudev et al., 1999).

The spent substrate of various *Pleurotus* species have been investigated for the removal of copper (Tay et al., 2010) and nickel-contaminated water (Tay et al., 2011), reduction of phenol content and toxicity in olive mill waste (Martirani et al., 1996), pesticides in effluents from the fruit packing industry (Karas et al., 2015), antibiotics in swine waste water (Chang et al., 2014), and textile dyes (Singh et al., 2011). *Lentinula edodes* spent substrate has been explored for treatment of acid mine drainage (Chang et al., 2000), effluents from olive mills (D'Annibale et al., 1998) and removal of cadmium (Chen et al., 2008). Laccase removal from waste spent substrate of *Ganoderma lucidum* has been tested for the removal of various toxic organic

compounds (Liao et al., 2012). Some mushroom species, although not specified in the article, have been investigated for fluoride removal from drinking water (Chen et al., 2015), sulfa antibiotics (Zhou et al., 2014a), and methylene blue from waste industrial waters (Yan and Wang, 2013; Yan et al., 2015).

20.3.3 Soil

Industrialization has advanced the quality of life. However, these advances have created by-products that may end up in soil, negatively affecting the quality of life that itself has created. *Agaricus bisporus* spent substrate/enzymes has been studied for its effect on distribution of zinc (Shuman, 1999a, 1999b), cadmium, and lead (Shuman, 1998) among soil fractions, the amelioration of zinc toxicity (Shuman and Li, 1997), the degradation of chlorophenols, polycyclic aromatic hydrocarbons, or aromatic monomers (Semple et al., 1995, 1998; Fermor et al, 2000; Li et al., 2010; García-Delgado et al., 2015), the inhibition of nitrification (Bazin et al., 1991), the treatment of hazardous wastes (Buswell, 1994), on stabilization of disturbed and commercial sites (Rupert, 1995), on volatile fatty acids bioproduction from waste activated sludge (Zhou et al., 2014b), and remediation of mining contaminated soils (Jordan et al., 2008a,b; Courtney et al., 2009; Frutos et al., 2010; Courtney and Harrington, 2012).

Pleurotus spp. spent substrate has been explored for degradation of polycyclic aromatic hydrocarbons (Eggen, 1999; Lau et al., 2003), removal/degradation of pentachlorophenol (Chiu et al., 1998) or petroleum (Chiu et al., 2009), and remediation of mining contaminated soils (Frutos et al., 2010). *Lentinula edodes* spent substrate has been evaluated for the removal/degradation of pentachlorophenol (Chiu et al., 1998).

20.3.4 Pesticides

Pesticide removal or degradation has been explored using *Agaricus bisporus* spent substrate for azoxystrobin (Herrero-Hernández et al., 2015), carbaryl, 1-naphthol (Kuo and Regan, 1992, 1999) and carbamate (Regan, 1994; Kuo and Regan, 1998), sorption and movement of atrazine and 2,4-D by soils (Baskaran et al., 1996), carbendazim and mancozeb (Ahlawat et al., 2010), chlorothalonil (Córdova Juárez et al., 2011), imidaclopridlinuron (Gao et al., 2015), diazinon and myclobutanil (Rodriguez-Cruz et al., 2012; Marin-Benito et al., 2014), tebuconazole (Herrero-Hernández et al., 2011), dimethoate, indoxacarb, buprofezin, terbuthylazine, metribuzin, metalaxyl-M, iprodione, azoxystrobin (Karanasios et al., 2010a), metalaxyl, benalaxyl, penconazole and tebuconazole, pyrimethanil, cyprodinil, iprovalicarb, and azoxystrobin (Marín-Benito et al., 2012).

Pleurotus spent substrate has been evaluated for degradation of various pesticides, including linuron, diazinon and myclobutanil (Rodriguez-Cruz et al., 2012; Marin-Benito et al., 2014), tebuconazole (Herrero-Hernández et al., 2011), metalaxyl, benalaxyl, penconazole and tebuconazole, pyrimethanil, cyprodinil, iprovalicarb, and azoxystrobin (Marín-Benito et al., 2012), chlorothalonil and imidaclopridlinuron (Gao et al., 2015), DDT (Purnomo et al., 2010), (Dimethoate 40 TC), IND (Steward 30 WG), BUP (Applaud 25 WP), TRB (Action puro 50 SC), MTR (Sencor 70 WG), MTX (Ridomil gold 48 SL), IPR (Rovral 50 SC), AZX (Quadris 25 SC), and CHL (Dursban 480 EC) (Karanasios et al., 2010b).

The spent substrate of *Lentinula edodes* has been assessed for degradation of metalaxyl, benalaxyl, penconazole, tebuconazole, pyrimethanil, cyprodinil, iprovalicarb, and azoxystrobin (Marín-Benito et al., 2012), and chlorothalonil and imidaclopridlinuron (Gao et al., 2015). Spent substrate from other species has been explored to degrade pesticides, for example, *Agaricus blazei* (*A. brasiliensis, A. subrufrescens*) for metsulfuron methyl (González-Matute et al., 2012).

20.4 Crop Production

Mushrooms are cultivated on organic substrates, naturally suitable for agricultural crops. The literature abounds on the various crops evaluated. Only a sampling is noted here.

One of the challenges with spent *Agaricus* mushroom substrate and growth of plants is the soluble salt content of fresh spent substrate may be excessive and affect plant growth. Chong and Rinker (1994a,b) determined from container plantings with trickle irrigation that the salts leach rapidly with no effect on the deciduous plant growth. Spent *Agaricus* compost leachate (Jarecki et al., 2005, 2012) can be used to supplement the nutrient requirements of plants.

20.4.1 Greenhouse Crops

Poole and Conorer (1974) proposed that *Agaricus bisporus* spent substrate be used as a potting soil. Since then SMS has been evaluated for the production of various greenhouse flowers and vegetables (Verdonck, 1984; Maher, 1991) such as chrysanthemum (Rathier, 1982), Easter lilies (White, 1976a,d; Dallon, 1987), *Helleborus* (Richter et al., 1980), marigolds (Rathier, 1982; Young et al., 2002), petunias (White, 1976c), poinsettia (White, 1976b), vegetable transplants (Lohr, 1983; Lohr et al., 1984a; Wang et al., 1984a; Lohr and Coffey, 1987), cucumbers (Celikel and Buyukalaca, 1999; Gonani et al., 2011), tomatoes (Rathier, 1982; Steffen et al., 1994, 1995a,b; Vavrina et al., 1996; Celikel and Tuncay, 1999a), and eggplant (Celikel and Tuncay, 1999b).

Agaricus subrufescens (syn. *A. blazei, A. brasiliensis*) spent substrate has been evaluated for production of lettuce seedling (Ribas et al., 2009; Marques et al., 2014) and *Lentinula edodes* spent substrate was examined for lettuce seedlings (Ribas et al., 2009).

20.4.2 Field Crops

One of the natural destinations for the large volumes of SMS is on land for agricultural crops. Spent substrate of *Agaricus bisporus* has been explored for the production of various vegetable crops, including asparagus, beetroot, cauliflower, cabbage, capsicums, celery, cucumber, lettuce, green gram, mustard, onion, potato, radish, snap bean, spinach, sugar beet, tomato, and zucchini (Anon., 1979; Male, 1981; Wang, 1983; Wang et al., 1984a,b; Schwank, 1985; Nguyen et al., 1987; Maynard, 1989, 1991, 1994b; Stephens et al., 1989; Faassen et al., 1992; Pill et al., 1993; Abak et al., 1994; Maher, 1994; Ranganathan and Selvaseelan, 1994, 1997a; Rhoads and Olson, 1995; Sochtig and Grabbe, 1995; Stewart, et al., 1998b, 1998c; Selvi and Selvaseelan, 1999; Maher et al., 2000; Medina et al., 2009; Kwack et al., 2012).

Pleurotus spp. spent substrate has been evaluated in interplantings with cabbage and eggplant (Abdallah et al., 2000), for cultivation of lettuce (Batista et al., 2000; Kwack et al., 2012), tomato, zucchini, pepper (Medina et al., 2009); in cucumber (Nguyen et al., 1987), corn (Adamović et al., 2007), and pepper production (Medina et al., 2009).

Lentinula edodes spent substrate has been tested for production of tomatoes (Lin and Chuen, 1993) and *Flammulina velutipes* spent substrate has been evaluated for production of honey dew melon seedlings (Van Tam and Wang, 2015). The fruit industry has an extensive land base and *Agaricus bisporus* spent substrate has been applied as a mulch to "Italian" prunes (Robbins et al., 1986), tea (Manivel et al., 1994), apples and apple seedlings (Koch, 1980; Delver, 1982; Delver and Wertheim, 1988; AntSaoir et al., 2000), and vineyards (Larrieta et al., 2010; Peregrina et al., 2012).

Large acreage crops have been evaluated with *Agaricus bisporus* spent substrate for field corn (Weber et al., 1997; Wuest and Fahy, 1991; Wuest et al., 1991, 1995), perennial rye grass, wheat (Maher, 1994; Maher et al., 2000), and barley (Courtney and Mullen, 2008).

20.4.3 General Soil Amendment/Fertilizer

Spent mushroom compost, in general, has been widely used as "manure" in India with great success (Sagar et al., 2009) and *Agaricus bisporus* is commercially being converted to a liquid fertilizer (*The Sarnia Journal*, 2014). *Agaricus* spent substrate has been co-composted with winery sludge (González-Marcos et al., 2015) for soil application. The influence of *Agaricus bisporus* spent substrate on soil physical and physicochemical properties has been noted by many authors (Robinson, 1988; Maher, 1990; Pryce, 1991; Gerrits, 1997b; Levanon and Danai, 1997; Ranganathan and Selvaseelan, 1997b,c; Maher et al., 2000; Medina et al., 2012). The physicochemical properties of *Pleurotus* spent substrate in a blend with *Agaricus* spent substrate has been reported by Medina et al. (2012). *Pleurotus* spent substrate has been evaluated as a biofertilizer (Zhu et al., 2013).

20.4.4 Nursery and Landscape

Eames (1977) was one of the first that proposed *Agaricus bisporus* spent substrate be used for production of shrubs. Many researchers have explored its use in nursery plant production (Henny, 1980; Smith, 1982; Chong et al., 1987, 1990, 1991a,b,c,d,e; Devonald, 1987; Chong and Wickware, 1989; Chong, 1991, 1999; Chong and Rinker, 1994a,b; Chong and Hamersma, 1996a,b; Raymond et al., 1998) or field grown foliage crops (Maynard, 1994c). Some investigators have pursued its usage in grass seed germination (Aamlid and Landschoot, 2007) or turf (Landschoot and McNitt, 1994).

Quimio et al. (1990) also explored the use *Pleurotus* spp. spent substrate in nursery crops.

20.5 Reuse in the Cultivation of Mushrooms

Mushroom spent substrate is not considered totally exhausted from the production of mushrooms. Thus, considerable research and practice have attempted to recycle the "spent" material in further mushroom production (Giménez, 2008).

20.5.1 Casing Material for *Agaricus bisporus*

Agaricus bisporus spent substrate has been investigated and used as a casing material for *Agaricus bisporus* as noted in the following: comparisons with peats and/or other local materials (Happ, 1974; Nair, 1976a,b; Stoller, 1979; Garcha and Sekhon, 1981; Nair and Bradley, 1981; Eicker and van Greuning, 1989; Shandilya, 1989a,b; Singh et al., 1992, 2000; Seaby, 1999; Pardo-Giménez and Pardo-González, 2008; Pardo-Giménez et al., 2010, 2011, 2012a,b), leaching experiments (Riahi et al., 1998) or treatment with chelating agents (Sharma et al., 1999), recomposting and leaching (Szmidt, 1994; Szmidt et al., 1995), handling and use (Kinrus, 1976; Wuest, 1976; Schisler and Wuest, 1982), separation and reuse of casing from spawn-run compost (Hesling, 1981; Nair and Bradley, 1981; Nair, 1985; Jablonsky and Srb, 1989). Additionally, it has been evaluated as a casing material for *A. bitroquis* (Guleria et al., 1989).

Composted spent sawdust media from *Pleurotus* cultivation has been tested as casing (Kim et al., 1998).

20.5.2 Cultivation Substrate for Mushrooms

Agaricus bisporus spent substrate has been re-used as the growing media for cultivation of 11 species (Flick, 1981), *Agaricus bisporus* (Till, 1963; Schisler, 1988; Rinker and Alm, 1990; Mamiro et al., 2007; Mamiro and Royse, 2008), *Auricularia* (Sharma and Jandaik, 1994),

Lentinula (Kilpatrick et al., 2000), *Pleurotus* (Mueller et al., 1984; Sharma and Jandaik, 1994), and *Volvariella* (Poppe, 2000).

Pleurotus spp. spent substrate has been recycled for the cultivation of *Pleurotus* spp. (Sharma and Jandaik, 1985, 1992; Shashirekha et al., 2002; Pardo-Giménez et al., 2012a,b), *Agaricus blazei* (Gern et al., 2010; González-Matute et al., 2011), and *Stropharia* (Poppe, 1995). *Lentinula edodes* spent substrate has been evaluated for the cultivation of *Pleurotus* (Royse, 1993) and *Volvariella* spent substrate for *Pleurotus* production (Quimio, 1988; Chang and Miles, 1989).

20.6 Food for Animals and Fish

Mushrooms are proteinaceous and the substrate formulations may include cereal straws and various grains that are components of animal diets. Thus, investigations have pursued the recycling of SMS as a feedstock. *Agaricus bisporus* spent substrate has had limited investigation (calves, Fazaeli et al., 2014; sheep, Wilson et al., 1983; dietary component for ruminants, Langar et al., 1982; Fazaeli and Masoodi, 2006; Ayala et al., 2011; Kim et al., 2011) for use in animals as a feed stock.

Pleurotus spp. spent substrate has been evaluated extensively as a feed stock with research including: cattle/ruminant feed (Kakkar et al., 1990; Adamović et al., 1998; Bae et al., 2006; Kim et al., 2011a,b, 2012); sugarcane bagasse compost in a dietary blend for ruminants (Permana, 1990; Zadrazil and Puniya, 1995); buffaloes (Bakshi et al., 1985; Kakkar and Dhanda, 1998); chickens (Azevedo et al., 2009), elk (Park et al., 2012a), goats (Park et al., 2012b), lambs and sheep (Calzada et al., 1987a,b) and degradation/silage studies (Zadrazil, 1977, 1980, 1984; Platt et al., 1981; Streeter et al., 1981; Bisaria and Madan, 1984; Sosulski and Coxworth, 1986; Zhang et al., 1995; Braun et al., 2000; Permana et al., 2000; Kim et al., 2008, 2010, 2014; Kwak et al., 2008, 2009; Xu et al., 2010). *Lentinula edodes* spent substrate has been evaluated to assist ruminant digestion (Yoshida et al., 1978; Zhang et al., 1995; Lin et al., 1998a,b; Braun et al., 2000).

Some other species spent substrate trials include *Volvariella volvacea* grown on rice straw or banana leaves for sheep (Sevilla et al., 1989), *Coprinus fimetarius* grown on rice and oat straws for goats (Mann et al., 1994), *Flammulina velutipes* for steers (Lee et al., 2006) and *Ganoderma balabacense* to assist milk production in dairy cattle (Liu et al., 2015).

20.6.1 Aquaculture

Some experiments have evaluated *Agaricus bisporus* spent substrate as a component in the diet of carp (*Cirrhina mirigala*) (Sehgal and Thomas, 1987; Sehgal and Simmi, 1991; Sehgal et al., 1993).

20.7 Pest Management

Uncomposted materials, composts, manures, and compost extracts have developed as alternatives to chemicals in the management of insects and diseases in agricultural and horticultural crops (Litterick et al., 2004). The spent substrate fits naturally into potential applications for pest control. SMSs have principally been investigated in disease management.

Agaricus bisporus spent substrate has been evaluated against Colorado potato beetles (Stoner et al., 1996; Gent et al., 1998) and as an organic alternative to methyl bromide in strawberries (Sances and Ingham, 1997).

20.7.1 Disease Management

Agaricus bisporus spent substrate, its extracts and composts (Borrero et al., 2013) have been assessed against various plant diseases, including apple scab (Yohalem et al., 1994, 1996; Cronin et al., 1996), damping-off and root rot of creeping bentgrass (Craft and Nelson, 1996), *Pythium* damping-off disease of tomatoes (Reigner et al., 2001), dry bubble disease in commercial mushrooms (Wuest et al., 1996; Guardino, 1998; Gea et al., 2012, 2014; Riahi et al., 2012), root-knot nematode (Verma, 1986, 1993; Kaul and Chhabra, 1993; Aslam and Saifullah, 2013); chili leaf and stem necrosis (Upadhyay, 2000); phytophthora in cucumber (Goonani et al. 2011), wilt of carnation and black root rot of cucumber (Ebben, 1980); suppression of turfgrass diseases (Viji et al., 2000), potato early dying disease caused by *Verticillium dahliae* and *Pratylenchus penetrans* (Gent et al., 1998; LaMondia et al., 1999), *Rhizoctonia* in cucumbers (Nguyen et al., 1987); *Fusarium* wilt of tomato (Harender et al., 1997), predaceous nematodes (Koning et al., 1996), and gas exchange in potatoes in the presence of *V. dahliae* or *P. penetrans* (Gent et al., 1999).

Pleurotus spent substrate has been screened against various diseases of cucumbers (Parada et al., 2012) and nematodes (Thorn and Barron, 1984; Hibbett and Thorn, 1994; Aslam and Saifullah, 2013) and spent substrate of *Lyophyllum decastes* (hatakeshimeji) against cucumber diseases (Parada, 2011a,b, 2012).

Lentinula edodes spent substrate has been evaluated against Rhizoctonia damping-off of cabbage (Huang, 1997; Huang and Huang, 2000) and diseases of tomato (Lin and Chuen, 1993).

20.8 Other Varied Uses

20.8.1 Renewable Energy

China produces about 70% of the world's mushrooms with most of its SMS burned (Zhu et al., 2013). *Agaricus bisporus* spent substrate has been evaluated as a biofuel (Kapu et al., 2012), alternative fuel (Maher et al., 2000; Williams et al., 2001; McCahey et al., 2003; Ryu et al., 2008 a,b; Finney et al., 2009 a,b,c) and in the production of biogas (Tumwasorn et al., 1980). *Pleurotus* spent substrate, too, has been examined as a source of biogas (Bisaria et al., 1983, 1990; Mehta et al., 1990). *Lentinula edodes* spent substrate has been tested as an alternative fuel (Pauli, 1999; Lee et al., 2008; Asada et al., 2011; Lin et al., 2015).

Agaricus bisporus spent substrate has been assessed as chars and activated carbon (Ma et al., 2014), airlift bioreactor to assess plant available nutrients (Velthof et al., 1998), heat resistant formulas (Donnelly and Busta, 1980), bedding for hogs (Durrell et al., 1997; Beattie et al., 2001), source of lignocellose-degrading enzymes (Ball and Jackson, 1995; note review by Phan and Sabaratnam, 2012), carrier material for preparation of bio-inoculants (Bahl and Jauhri, 1986; Bahl et al., 1989), insulation factor for bricks (Muñoz Velascoa et al., 2014), strengthening material for concrete (Russell et al., 2005) and in vermiculture (Edwards et al., 1985; Tajbakhsh et al., 2008).

Agaricus waste mushroom tissue has potential for production of phenoloxidases (Steffen and Grabbe, 1995), human dietary nutrients (Anon., 2009) and as a diet component for chickens (Giannenas et al., 2010) and turkeys (Giannenas et al., 2011). *Pleurotus* has been evaluated for extracellular enzyme production (Tan and Wahab, 1997; note review by Phan and Sabaratnam, 2012), nanoparticle production (Vigneshwaran et al., 2007), production of *Lactococcus lactis* used in cheese and buttermilk production (Wu et al., 2014), and super absorbent resins (Ding and Gong, 2013). *Lentinula edodes* spent substrate has been used in vermiculture (Pauli, 1999).

Flammulina spent substrate has been explored for the coproduction of the biological pesticide *Bacillus thuringiensis* (Wu et al., 2013, 2014). Other species of spent substrate have been used as animal feed (Sova and Cibulka, 1980) and extraction of cellulolytic bacteria from *Volvariella volvacea* (Wong et al., 1990).

20.9 Conclusion

Commercial mushroom production produces a coproduct of great value. Researchers have addressed the possible usages for many decades, exploring its resource potential. Its utilization will not be restricted to a single application but will only be limited by the resourcefulness and ingenuity of mushroom farmers, scientists, and entrepreneurs.

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21

Chemical, Nutritional, and Bioactive Potential of Mushrooms

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21.1 Brief Introduction

Edible mushrooms are widely consumed in many countries as a food having a high commercial and culinary value, mainly due to organoleptic properties, such as texture and flavor, and it is possible to distinguish a mushroom species based on its characteristic odor or flavor (Guillamón et al., 2010a). Described as rich in minerals, water, proteins, essential and non-essential amino acids, carbohydrates, and poor in lipids, mushrooms are highly nutritious; mushroom proteins are considered of higher nutritional quality than those of vegetables, being comparable to proteins of animal origin such as meat, eggs, and milk (Wang et al., 2014).

There are available many studies showing that mushrooms have a wide variety of biomolecules with medicinal properties, with great value in the prevention and treatment of various diseases, being recognized as functional foods and as a source for the development of pharmaceuticals and nutraceuticals (Newman et al., 2000; Ferreira et al., 2009, 2010; Barreira et al., 2014; Gyawali et al., 2014). Mushrooms' bioactive molecules are responsible for their antioxidant (Ferreira et al., 2009), antitumor (Ferreira et al., 2010), antibacterial (Alves et al., 2012), immunostimulatory, antifibrotic, anti-inflammatory (Kohno et al., 2008), antiviral (Barreira et al., 2014), antifungal (Alves et al., 2013), anti-atherosclerotic (Guillamón et al., 2010b; De Silva et al., 2012), hypoallergenic, antiatherogenic, hypoglycemic, hepatoprotective (Nitha et al., 2013), and hypotensive (Guillamón et al., 2010b) properties.

According to the available reports on mushrooms' bioactivities, the refined extracts obtained from the mycelium or fruiting body, that can be consumed in the form of tablets or capsules as dietary supplements and have potential therapeutic application, are considered nutraceuticals, products derived from food sources that provide nutritional value but also provide extra health benefits (Chang and Buswell, 1996).

In this chapter, the chemical composition, nutritional value, and bioactive properties of mushrooms species are reviewed and will be highlighted in the following sections.

21.2 Chemical Composition and Nutritional Properties

The chemical and nutritional composition of wild and cultivated mushrooms are presented in Tables 21.1–21.5.

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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Regarding the chemical composition of mushrooms, water content is the parameter with the greatest variability, due to different growth and climatic conditions that may influence their fruiting, depending also on the species and on collection and storage procedures. Moisture content varies between 65 and 90 g/100 g fresh weight (fw), except for two *Bovista* species, namely *B. nigrescens* (Pers.) and *B. aestivalis* (Bonord.) Demoulin (Pereira et al., 2012), that gives 16 to 23 g moisture per 100 g fw, respectively. The moisture is easily lost after harvest, due to evaporation; consequently, the chemical composition of mushrooms varies within and between species, and corresponding maturation. Therefore, the dry matter content of mushrooms is relatively low, about 10 to 30 g/100 g fw and it is mainly formed by carbohydrates, lipids, proteins, and ash; low lipid concentrations result in low energy values. The mushrooms provide small amounts of lipids (Table 21.1) ranging from 0.11 g/100 g dry weight (dw) in *Agaricus campestres* (L.) (Pereira et al., 2012) to 8.30 g/100 g dw in *Amanita mairei* Foley (Leal et al., 2013), for example.

The content of crude protein depends not only on the environment and maturation stage, but also on the species. The informative data on mushroom crude protein is provided in Table 21.1. High levels are found in edible species and can vary from 3.87 g/100 g dw in *Flammulina velutipes* (Curtis) Singer cultivated species (Reis et al., 2012) to 76.63 g/100 g dw in *Lepista inversa* (Scop. Fr.) Pat. wild species (Heleno et al., 2009). Some species, such as *Tricholoma imbricatum* (Fr.) P. Kumm., *Russula delica* Fr., *Laccaria laccata* (scop.: Fr.) Berk. & Broome, *Cortinarius glaucopus* (Schaeff) and *Fistulina hepatica* Schaeff.: Fr. (Heleno et al., 2009) showed protein contents between 50.09 and 63.69 g/100 g dw (Table 21.1).

The ash content gives us a general idea about the mineral content of mushrooms and usually ranges from 3.5 g/100 g dw in *A. campestris* (Beluhan et al., 2011) to 32.10 g/100 g dw in *Gyromitra esculenta* (Pers. ex Pers.) Fr. (Leal et al., 2013). The content of carbohydrates varies between 10.35 g/100 g dw in *L. inversa* and 88.79 g/100 g dw in *Boletus regius* Krombh (Leal et al., 2013), some heterogeneity between species is also observed.

The energy value ranges between 258.84 kcal/100 g dw in *Russula olivacea* (Schaeff.) Fr. (Grangeia et al., 2011) and 413.46 kcal/100 g dw in *Craterellus cornucopioides* (L.: Fr.) Pers. (Beluhan et al., 2011) (Table 21.1).

The major fatty acids described in mushrooms are linoleic acid (C18:2n6) and oleic acid (C18: 1n9) and, in general, unsaturated fatty acids predominate over saturated fatty acids, except for *Hericium erinaceum* (Bull.) Persoon (Heleno et al., 2015a), *Hypholoma capnoides* (Fr.) Quel., *Lactarius salmonicolor* R. Heim & Leclair (Heleno et al., 2009), *Lactarius citriolens* Pouzar (Vieira et al., 2014), and *Lycoperdon umbrinum* Pers. (Pereira et al., 2014) that showed higher levels of saturated fatty acids (Table 21.2).

Mannitol and trehalose are the major sugars identified in mushrooms, despite the different profiles found in several species (Table 21.3). Typically, mannitol participates in the growth and firmness of the fruit bodies and varies from species to species (Kalač, 2009). Mannitol was the most abundant sugar in wild species and varied between 11.03 g/100 g dw in *Lyophyllum decastes* (Fries: Fries) Singer and 43.34 g/100 g dw in *Clavariadelphus truncatus* (Quél.) Donk (Pereira et al., 2012). The cultivated species *Agaricus bisporus* (J.E. Lange) Imbach- white, *A. bisporus-* brown and *Lentinula edodes* (Berk.) Pegler had the highest concentration of mannitol: 64.15 g/100 g dw, 47.97 g/100 g dw, and 49.51 g/100 g dw, respectively (Reis et al., 2012).

Trehalose prevailed in *Pleurotus eryngii* (DC.) Quél. (Reis et al., 2014) and *Chlorophyllum rhacodes* (Vittadini) Vellinga (Pereira et al., 2012) with intervals between 14.21 g/100 g dw and 25.57 g/100 g dw. The level of trehalose in the cultivated sample of *P. eryngii* was 72.82 g/100 g dw (Reis et al., 2012). *Coprinus comatus* (O.F.Müll.) Pers (Vaz et al., 2011) and *Cortinarius praestans* Cordier (Pereira et al., 2012) revealed 42.82 and 60.51 g/100 g dw, respectively. Small amounts of other sugars were also described, in particular, arabinose, fructose, mannose, glucose, sucrose, and melezitose (Table 21.3).

Table 21.1 Approximate composition and energy values of wild and cultivated mushroom species.

Mushroom Species	Moisture (g/100 g fw)	Crude Fat (g/100 g dw)	Crude Protein (g/100 g dw)	Ash (g/100 g dw)	Carbohydrates (g/100 g dw)	Energy (kcal/100 g dw)	Reference
Agaricus albertii Bon	90.73 ± 0.24	1.38 ± 0.06	19.83 ± 0.03	22.13 ± 0.71	56.66 ± 0.54	318.36 ± 1.78	Reis et al., 2014
Agaricus bisporus (J.E. Lange) Imbach (white, cultivated)	91.27 ± 0.45	2.18 ± 0.34	14.09 ± 0.23	9.74 ± 1.95	73.80 ± 6.53	353.49 ± 22.68	Reis et al., 2012
Agaricus bisporus (J.E. Lange) Imbach (brown, cultivated)	91.64 ± 0.99	1.67 ± 0.24	15.43 ± 0.48	11.36 ± 0.24	71.53 ± 12.32	345.10 ± 44.38	Reis et al., 2012
Agaricus campestres (L.)	nd	2.7 ± 0.56	38.89 ± 0.67	3.5 ± 0.66	nd	375.13 ± 0.24	Beluhan et al., 2011
	88.17 ± 0.44	0.11 ± 0.00	18.57 ± 0.00	23.16 ± 0.00	58.16 ± 0.00	307.91 ± 0.00	Pereira et al., 2012
Agaricus comtulus Fries	87.94 ± 0.77	0.46 ± 0.00	21.29 ± 0.83	28.14 ± 0.18	50.11 ± 0.89	289.74 ± 0.52	Pereira et al., 2012
Agaricus excellens (F.H. Møller) Nauta	87.72 ± 0.26	1.37 ± 0.23	14.47 ± 0.61	29.64 ± 1.82	54.52 ± 1.83	288.29 ± 4.36	Reis et al., 2014
Agaricus lutosus (Møller) Møller	87.04 ± 2.01	1.10 ± 0.04	23.24 ± 0.44	25.96 ± 2.64	49.71 ± 1.72	301.67 ± 7.31	Pereira et al., 2012
Amanita caesarea (Scop.) Pers.		3.50 ± 0.00	34.77 ± 0.06	6.05 ± 0.01	55.63 ± 0.06	nd	Ouzouni et al., 2009
	94 ± 1	6.4 ± 0.1	6.3 ± 0.1	14.8 ± 0.1	72.5 ± 0.3	373 ± 1	Fernandes et al, 2015
Amanita crócea (Quél. in Bourd.) Singer ex Singer	89.04 ± 0.00	4.62 ± 0.16	20.02 ± 1.33	25.73 ± 1.75	49.64 ± 1.34	320.19 ± 4.39	Leal et al., 2013
Amanita curtipes E.-J.Gilbert	80 ± 1	8.6 ± 0.3	6.4 ± 0.4	17.2 ± 0.1	67.8 ± 0.4	374 ± 2	Fernandes et al., 2015
Amanita mairei Foley	76.82 ± 8.50	8.30 ± 0.00	17.74 ± 0.79	11.21 ± 0.09	62.75 ± 0.74	396.67 ± 0.26	Leal et al., 2013
Amanita umbrinolutea (Secr. ex Gillet)	73.60 ± 0.17	6.77 ± 0.00	16.78 ± 0.00	28.86 ± 0.00	47.59 ± 0.00	318.41 ± 0.01	Pereira et al., 2012
Armillaria mellea (Vahl) P. Kumm	88.27 ± 0.60	5.56 ± 0.53	16.38 ± 1.34	6.78 ± 1.28	71.28 ± 1.06	400.68 ± 5.50	Vaz et al., 2011
	nd	2.10 ± 0.02	24.47 ± 0.12	7.95 ± 0.02	65.47 ± 0.15	nd	Ouzouni et al., 2009
Armillaria tabescens (Scop.) Emel	nd	2.54 ± 0.03	22.90 ± 0.20	7.63 ± 0.15	66.87 ± 0.06	nd	Ouzouni et al., 2009
Boletus armeniacus (Quél.)	71.50 ± 0.43	1.56 ± 0.42	18.25 ± 0.06	12.09 ± 0.35	68.10 ± 0.51	359.45 ± 0.52	Pereira et al., 2012
Boletus aureus Schaeff.	nd	4.47 ± 0.02	27.17 ± 0.15	6.25 ± 0.02	62.10 ± 0.10	nd	Ouzouni et al., 2009
Boletus edulis Bull. Fr.	nd	2.92 ± 0.41	36.91 ± 0.02	5.3 ± 0.87	nd	355.66 ± 0.18	Beluhan et al., 2011
	93 ± 1	4.3 ± 0.3	23 ± 2	7.9 ± 0.1	65 ± 2	390 ± 2	Fernandes et al., 2013a
	91 ± 1	5.0 ± 0.4	16.4 ± 0.1	8.0 ± 0.1	71 ± 1	375 ± 2	Fernandes et al., 2014
	nd	2.23 ± 0.02	10.65 ± 0.47	5.26 ± 0.44	81.86 ± 0.41	390.09 ± 1.32	Heleno et al., 2015c

(Continued)

Table 21.1 (Continued)

Mushroom Species	Moisture (g/100 g fw)	Crude Fat (g/100 g dw)	Crude Protein (g/100 g dw)	Ash (g/100 g dw)	Carbohydrates (g/100 g dw)	Energy (kcal/100 g dw)	Reference
Boletus erythropus (Pers.)	88.36 ± 1.49	0.75 ± 0.02	20.92 ± 0.05	25.90 ± 0.28	52.44 ± 0.20	300.15 ± 0.88	Grangeia et al., 2011
Boletus fragrans (Vittadini)	77.99 ± 0.07	1.83 ± 0.17	17.15 ± 0.04	4.74 ± 0.19	76.29 ± 0.27	390.19 ± 0.06	Grangeia et al., 2011
Boletus impolitus Fr.	88.90 ± 1.45	2.94 ± 0.33	16.01 ± 0.02	24.43 ± 0.84	56.63 ± 0.84	316.98 ± 1.21	Pereira et al., 2012
Boletus porosporus (Imler ex Bon & G. Moreno)	65.57 ± 7.94	0.96 ± 0.06	15.74 ± 1.78	4.20 ± 0.05	79.11 ± 1.82	388.01 ± 0.09	Leal et al., 2013
Boletus regius Krombh.	79.15 ± 9.43	1.59 ± 0.11	5.22 ± 0.22	4.40 ± 0.29	88.79 ± 0.44	390.36 ± 0.42	Leal et al., 2013
Bovista aestivalis (Bonord.) Demoulin	23.23 ± 0.93	0.18 ± 0.02	15.59 ± 1.23	31.86 ± 0.20	52.37 ± 1.31	273.44 ± 0.49	Pereira et al., 2012
Bovista nigrescens (Pers.)	16.41 ± 0.18	3.64 ± 0.96	20.94 ± 0.31	3.24 ± 0.17	72.18 ± 0.76	405.24 ± 3.88	Pereira et al., 2012
Calocybe gambosa (Fr.) Donk	nd	1.34 ± 0.12	36.65 ± 0.34	7.98 ± 0.79	nd	334.05 ± 0.29	Beluhan et al., 2011
	90.92 ± 1.08	0.83 ± 0.11	15.46 ± 0.24	13.89 ± 1.41	69.83 ± 1.22	348.58 ± 3.58	Vaz et al., 2011
Calvatia utriformis (Bull.) Jaap.	78.00 ± 1.36	1.90 ± 0.01	20.37 ± 0.49	17.81 ± 0.22	59.91 ± 0.40	338.26 ± 0.61	Grangeia et al., 2011
Cantharellus cibarius Fr.	nd	2.88 ± 0.02	21.57 ± 0.21	9.44 ± 0.01	66.07 ± 0.23	nd	Ouzouni et al., 2009
	nd	1.9 ± 0.61	30.91 ± 0.28	8.8 ± 0.05	nd	355.70 ± 0.28	Beluhan et al., 2011
Catathelasma ventricosum (Pk.) Singer	89.62 ± 2.07	1.52 ± 0.55	29.13 ± 0.65	12.59 ± 0.62	56.76 ± 2.04	362.42 ± 4.55	Liu et al., 2012a
Chlorophyllum rhacodes (Vittadini) Vellinga	88.28 ± 0.33	3.29 ± 0.33	19.32 ± 0.04	12.10 ± 0.31	65.29 ± 0.48	368.03 ± 0.30	Pereira et al., 2012
Clavariadelphus pistillaris L.:Fr. Donk	84.22 ± 1.78	0.59 ± 0.07	16.27 ± 0.24	20.77 ± 0.86	62.37 ± 0.48	319.88 ± 2.67	Pereira et al., 2012
Clavariadelphus truncatus (Quél.) Donk	90.97 ± 1.29	1.54 ± 0.25	15.98 ± 0.15	12.86 ± 0.33	69.62 ± 0.37	356.26 ± 0.02	Pereira et al., 2012
Clitocybe costata Kühner & Romagn	76.92 ± 2.11	1.50 ± 0.00	17.27 ± 0.25	10.87 ± 1.36	70.36 ± 1.10	364.02 ± 3.84	Pereira et al., 2012
Clitocybe gibba (Pers.) Kumm	72.66 ± 0.99	4.29 ± 0.00	14.59 ± 0.27	20.68 ± 0.15	60.45 ± 0.23	338.74 ± 0.42	Pereira et al., 2012
Clitocybe odora (Fr.) P. Kumm.	88.49 ± 3.03	2.46 ± 0.04	17.33 ± 1.37	9.55 ± 0.68	70.66 ± 1.09	374.12 ± 1.81	Vaz et al., 2011
Clitocybe subconnexa (Murrill)	nd	1.02 ± 0.09	7.42 ± 0.25	5.98 ± 0.04	27.35 ± 0.13	381.18 ± 0.23	Heleno et al., 2015b
Clitocybe maxima (Gartn. ex Mey.: Fr.) Quél	90.81 ± 0.79	6.02 ± 0.07	25.93 ± 3.02	4.93 ± 0.25	63.12 ± 1.43	415.05 ± 3.28	Liu et al., 2012a
Clitopilus prunulus (Scop. ex Fr.) P. Kumm	89.78 ± 1.46	1.01 ± 0.06	18.13 ± 0.37	30.19 ± 2.50	50.66 ± 2.21	284.30 ± 5.80	Grangeia et al., 2011
Coprinus comatus (O.F.Müll.) Pers.	85.19 ± 0.50	1.13 ± 0.05	15.67 ± 0.23	12.85 ± 0.42	70.36 ± 0.26	354.27 ± 1.18	Vaz et al., 2011
	nd	1.80 ± 0.10	11.84 ± 0.25	10.07 ± 0.57	76.29 ± 0.41	368.72 ± 1.27	Stojković et al., 2013a

Coprinus comatus (O.F.Müll.) Pers (cultivated)	nd	1.98 ± 0.00	10.98 ± 0.32	10.46 ± 0.17	76.57 ± 0.26	368.08 ± 0.47	Stojković et al., 2013a
Cortinarius glaucopus (Schaeff)	91.67 ± 0.31	1.89 ± 0.15	50.09 ± 0.65	16.40 ± 0.12c	31.62 ± 0.73	343.84 ± 0.05	Heleno et al., 2009
Cortinarius praestans Cordier	89.16 ± 0.19	2.58 ± 0.28	14.56 ± 0.24	18.89 ± 0.01	63.98 ± 0.22	337. 34 ± 1.00	Pereira et al., 2012
Craterellus cornucopioides (L.: Fr.) Pers.	nd	4.87 ± 0.21	47.21 ± 0.34	10.08 ± 0.83	nd	413.46 ± 0.14	Beluhan et al., 2011
	87.19 ± 2.63	5.87 ± 0.14	21.04 ± 2.69	8.37 ± 0.66	64.72 ± 2.31	412.55 ± 5.56	Liu et al., 2012a
Entoloma clypeatum (L.) P. Kumm.	nd	6.21 ± 0.44	27.98 ± 0.21	9.21 ± 0.91	nd	374.80 ± 0.19	Beluhan et al., 2011
Fistulina hepatica Schaeff.: Fr.	87.51 ± 0.98	2.63 ± 0.49	63.69 ± 1.16	11.30 ± 0.53	22.98 ± 0.43	364.98 ± 2.16	Heleno et al., 2009
		3.17 ± 0.02	22.60 ± 0.20	8.20 ± 0.10	66.00 ± 0.10	nd	Ouzouni et al., 2009
Flammulina velutipes (Curtis) Singer	nd	6.45 ± 0.43	27.95 ± 0.34	7.39 ± 0.56	nd	343.69 ± 0.16	Beluhan et al., 2011
	90.68 ± 0.58	1.84 ± 0.14	17.89 ± 0.02	9.42 ± 0.66	70.85 ± 0.36	371.53 ± 2.36	Pereira et al., 2012
Flammulina velutipes (Curtis) Singer (cultivated)	87.87 ± 1.33	1.73 ± 0.33	3.87 ± 0.08	7.25 ± 1.07	87.14 ± 10.39	357.87 ± 37.02	Reis et al., 2012
Ganoderma lucidum (Curtis) P. Karst	nd	4.43 ± 0.00	11.34 ± 1.21	2.80 ± 0.01	81.48 ± 1.11	410.93 ± 0.04	Stojković et al., 2013b
Ganoderma lucidum (Curtis) P. Karst (cultivated)	nd	3.72 ± 0.00	9.93 ± 0.26	8.19 ± 0.10	78.16 ± 0.21	385.86 ± 0.29	Stojković et al., 2013b
Gyromitra esculenta (Pers. ex Pers.) Fr.	85.68 ± 8.39	0.73 ± 0.01	14.74 ± 0.79	32.10 ± 3.55	52.43 ± 2.22	275.23 ± 10.07	Leal et al., 2013
Helvella lacunosa (Afzel.)	82.37 ± 3.43	2.40 ± 0.01	4.40 ± 0.36	21.70 ± 1.10	71.50 ± 1.02	325.21 ± 3.05	Leal et al., 2013
Hericium coralloides (Scop.) Pers.	nd	2.38 ± 0.14	7.25 ± 0.15	9.31 ± 0.47	81.06 ± 0.34	374.67 ± 1.84	Heleno et al., 2015a
Hericium erinaceum (Bull.) Persoon	nd	1.75 ± 0.27	15.40 ± 0.38	3.49 ± 0.20	79.36 ± 0.32	394.79 ± 0.95	Heleno et al., 2015a
Hygrophoropsis aurantiaca (Wulfen) Maire	84.59 ± 1.27	2.20 ± 0.11	36.40 ± 0.60	5.92 ± 0.06	55.48 ± 0.52	387.32 ± 0.25	Heleno et al., 2009
Hygrophorus chrysodon (Fr.) Fr.	92.09 ± 1.01	3.48 ± 0.09	15.11 ± 0.18	26.91 ± 1.99	54.51 ± 1.28	309.74 ± 5.97	Pereira et al., 2012
Hygrophorus pustulatus (Persoon : Fries) Fries	93.03 ± 0.79	3.06 ± 0.51	18.64 ± 0.40	14.04 ± 0.14	64.26 ± 0.72	359.16 ± 1.40	Grangeia et al., 2011
Hygrophorus russula (Schaeff.:Fr.) Quélet.		6.00 ± 0.10	32.47 ± 0.06	8.18 ± 0.02	53.33 ± 0.06	nd	Ouzouni et al., 2009
Hypholoma capnoides (Fr.) Quel.	83.57 ± 2.08	0.36 ± 0.05	36.36 ± 0.21	28.29 ± 2.51	34.99 ± 2.42	288.64 ± 10.05	Heleno et al., 2009
Laccaria amethystea (Bull. ex Gray) Murr.	86.13 ± 0.64	2.78 ± 0.05	29.84 ± 2.30	10.31 ± 0.92	57.07 ± 1.92	377.70 ± 3.64	Liu et al., 2012a
Laccaria laccata (Scop.) Cooke	88.25 ± 1.86	3.76 ± 0.58	62.78 ± 1.07	20.69 ± 1.50	12.77 ± 0.78	336.08 ± 6.88	Heleno et al., 2009
Lactarius citriolens Pouzar	nd	5.37 ± 0.30	10.89 ± 0.33	6.99 ± 0.23	76.76 ± 0.35	398.89 ± 1.74	Vieira et al., 2014

(Continued)

Table 21.1 (Continued)

Mushroom Species	Moisture (g/100 g fw)	Crude Fat (g/100 g dw)	Crude Protein (g/100 g dw)	Ash (g/100 g dw)	Carbohydrates (g/100 g dw)	Energy (kcal/100 g dw)	Reference
Lactarius salmonicolor R.Heim & Leclair	87.72 ± 0.94	2.03 ± 0.36	37.28 ± 0.11	23.28 ± 1.41	37.41 ± 1.42	317.05 ± 5.85	Heleno et al., 2009
Lactarius turpis (Weinm.) Fr.	nd	2.06 ± 0.27	13.06 ± 0.29	7.21 ± 0.12	77.68 ± 0.35	381.47 ± 1.29	Vieira et al., 2014
Lentinula edodes (Berk.) Pegler (cultivated)	79.78 ± 1.31	1.73 ± 0.09	4.40 ± 0.45	6.72 ± 0.25	87.14 ± 6.38	359.99 ± 24.63	Reis et al., 2012
Lentinus edodes (Berk.) Pegler (cultivated)	nd	1.14 ± 0.01	16.0 ± 0.05	6.24 ± 0.05	76.62 ± 0.07	380.74 ± 0.17	Heleno et al., 2015c
Lepista inversa (Scop. Fr.) Pat.	87.73 ± 1.01	2.48 ± 0.21	76.63 ± 0.46	10.54 ± 0.07	10.35 ± 0.45	370.24 ± 0.77	Heleno et al., 2009
Lepista nuda (Bull.) Cooke	nd	3.23 ± 0.01	34.37 ± 0.15	6.03 ± 0.02	56.33 ± 0.15	nd	Ouzouni et al., 2009
Leucoagaricus leucothites Vittad. Wasser	85.29 ± 1.00	1.10 ± 0.15	20.51 ± 0.47	26.46 ± 0.01	51.93 ± 0.53	299.64 ± 0.57	Pereira et al., 2012
Lyophyllum decastes (Fries: Fries) Singer	87.38 ± 1.40	2.10 ± 0.12	25.52 ± 3.49	7.38 ± 0.64	64.99 ± 2.96	380.98 ± 1.82	Grangeia et al., 2011
Lycoperdon echinatum Pers.	85.24 ± 0.48	1.22 ± 0.20	23.52 ± 2.20	9.43 ± 0.23	65.83 ± 2.09	368.34 ± 0.66	Grangeia et al., 2011
Lycoperdon umbrinum Pers.	71.98 ± 0.32	0.37 ± 0.00	14.53 ± 0.07	33.14 ± 1.06	51.96 ± 0.70	269.29 ± 3.00	Pereira et al., 2012
Macrolepiota excoriata (Schaeff.) M.M. Moser	88.92 ± 1.57	1.55 ± 0.10	25.28 ± 2.64	28.98 ± 1.11	44.19 ± 2.14	291.84 ± 3.51	Grangeia et al., 2011
Macrolepiota procera (Scop.) Singer	nd	2.23 ± 0.22	24.22 ± 0.67	5.37 ± 0.04	nd	389.46 ± 0.14	Beluhan et al., 2011
	85.9 ± 0.3	2.9 ± 0.1	19 ± 1	8.0 ± 0.2	70 ± 1	383 ± 1	Fernandes et al., 2013b
Morchella elata Fr.	nd	3.91 ± 0.77	35.75 ± 0.99	9.01 ± 0.05	nd	358.52 ± 0.44	Beluhan et al., 2011
Morchella esculenta (L.) Pers.	90.79 ± 8.57	2.59 ± 0.42	11.52 ± 0.89	11.34 ± 0.8	74.55 ± 0.87	367.59 ± 0.78	Heleno et al., 2013a
Pleurotus eryngii (DC.) Quél.	89.00 ± 1.39	1.45 ± 0.27	11.00 ± 0.18	6.18 ± 0.55	81.36 ± 9.45	362.18 ± 34.00	Reis et al., 2012
Pleurotus eryngii (DC.) Quél. (cultivated)	82.59 ± 0.36	4.36 ± 0.14	2.09 ± 0.01	14.95 ± 0.91	78.60 ± 0.75	362.00 ± 2.06	Reis et al., 2014
Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm.	nd	2.08 ± 0.06	24.90 ± 0.89	7.62 ± 0.23	nd	370.99 ± 0.16	Beluhan et al., 2011
Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm. (cultivated)	89.17 ± 2.12	1.39 ± 0.18	7.02 ± 0.55	5.72 ± 0.74	85.87 ± 19.21	362.60 ± 71.65	Reis et al., 2012
Ramaria aurea (Schaeff.) Quél.	88.52 ± 0.12	2.26 ± 0.05	14.60 ± 0.10	5.68 ± 0.74	77.47 ± 0.61	388.58 ± 1.93	Pereira et al., 2012
Ramaria largentii Marr & D.E. Stuntz	nd	5.67 ± 0.12	28.80 ± 0.46	6.67 ± 0.12	58.87 ± 0.25	nd	Ouzouni et al., 2009

Russula aurea Pers.	79.99 ± 9.13	1.24 ± 0.02	10.33 ± 10.33	12.75 ± 0.38	75.68 ± 0.79	355.18 ± 1.01	Leal et al., 2013
Russula delica Fr.	86.69 ± 0.73	0.91 ± 0.16	50.59 ± 1.02	22.93 ± 2.16	25.57 ± 1.32	312.81 ± 9.15	Heleno et al., 2009
	nd	4.44 ± 0.04	26.10 ± 0.30	5.61 ± 0.03	63.87 ± 0.31	nd	Ouzouni et al., 2009
	92 ± 1	3.4 ± 0.2	13.8 ± 0.5	8.8 ± 0.4	74 ± 1	363 ± 2	Fernandes et al., 2014
Russula cyanoxantha (Schaeff.) Fr.	85.44 ± 0.99	1.52 ± 0.52	16.80 ± 0.06	7.03 ± 0.87	74.65 ± 1.01	379.46 ± 0.61	Grangeia et al., 2011
Russula olivacea (Schaeff.) Fr.	84.58 ± 1.01	1.99 ± 0.44	16.84 ± 0.05	37.78 ± 5.20	43.38 ± 3.71	258.84 ± 14.71	Grangeia et al., 2011
Russula virescens (Schaeff.) Fr.	92.49 ± 4.81	1.85 ± 0.09	21.85 ± 0.79	11.04 ± 0.19	62.27 ± 0.83	365.09 ± 0.87	Leal et al., 2013
Stropharia rugoso-annulata Farlow apud Murr.	88.04 ± 1.37	3.72 ± 0.24	25.89 ± 1.72	6.04 ± 0.34	64.35 ± 1.32	399.55 ± 2.79	Liu et al., 2012a
Suillus mediterraneensis (Jacquetant & Blum) Redeuilh	91.20 ± 1.85	2.61 ± 0.49	24.32 ± 0.35	27.64 ± 0.80	45.42 ± 1.34	302.48 ± 1.49	Heleno et al., 2009
Suillus variegates (Sw.) Kuntze	90.77 ± 0.76	3.31 ± 0.49	17.57 ± 0.56	15.36 ± 2.10	63.76 ± 2.17	355.12 ± 4.19	Pereira et al., 2012
Tricholoma matsutake (S. Itô et S. Imai) Singer	nd	5.04 ± 0.01	20.30 ± 0.02	8.89 ± 0.05	36.67 ± 0.04	nd	Liu et al., 2010
Tricholoma imbricatum (Fr.) P.Kumm.	82.42 ± 1.15	1.88 ± 0.11	50.45 ± 0.83	6.45 ± 0.27	41.21 ± 0.56	383.61 ± 1.44	Heleno et al., 2009
Xerocomus badius (Fr.) E.-J.Gilbert	nd	4.22 ± 0.03	8.08 ± 0.14	7.32 ± 0.02	80.38 ± 0.15	391.83 ± 0.04	Heleno et al., 2015c
Volvopluteus gloiocephalus (DC.) Vizzini, Contu & Justo	nd	4.62 ± 0.04	19.66 ± 0.14	14.19 ± 0.07	13.97 ± 0.34	366.34 ± 0.05	Heleno et al., 2015b

nd, not detected; fw, fresh weight; dw, dried weight.

Table 21.2 Main fatty acids (relative percentages) found in wild and cultivated mushroom species.

Mushroom Species	Palmitic Acid (C16:0)	Stearic Acid (C18:0)	Oleic Acid (C18:1n9)	Linoleic Acid (C18:2n6)	Total SFA	Total MUFA	Total PUFA	Reference
Agaricus albertii (Bon.)	11.14 ± 0.07	3.12 ± 0.04	2.05 ± 0.60	75.75 ± 0.51	21.14 ± 0.06	2.39 ± 0.61	76.47 ± 0.55	Reis et al., 2014
Agaricus arvensis Schaeff	11.1 ± 0.48	2.83 ± 0.26	2.53 ± 0.46	78.0 ± 1.45	16.6	3.61	78.8	Ayaz et al., 2011
Agaricus bisporus (J.E. Lange) Imbach (white, cultivated)	11.9 ± 0.3	3.1 ± 0.0	1.1 ± 0.6	77.7 ± 0.7	20.3 ± 0.1	1.4 ± 0.6	78.3 ± 0.7	Reis et al., 2012
Agaricus bisporus (J.E. Lange) Imbach (brown, cultivated)	11.1 ± 0.1	3.0 ± 0.2	1.2 ± 0.4	79.4 ± 0.2	18.4 ± 0.6	1.8 ± 0.4	79.8 ± 0.2	Reis et al., 2012
Agaricus campestres (L.)	12.48 ± 0.01	2.73 ± 0.01	6.09 ± 0.01	68.97 ± 0.07	20.91 ± 0.05	9.05 ± 0.03	70.04 ± 0.02	Pereira et al., 2012
Agaricus comtulus Fries	12.98 ± 0.35	2.66 ± 0.03	3.50 ± 0.01	72.88 ± 0.57	22.04 ± 0.63	4.42 ± 0.04	73.55 ± 0.59	Pereira et al., 2012
Agaricus excellens (F.H. Møller) Nauta	14.88 ± 0.31	3.57 ± 0.02	5.47 ± 0.55	51.21 ± 1.09	28.79 ± 0.13	19.16 ± 0.97	52.05 ± 1.10	Reis et al., 2014
Agaricus lutosus (Møller) Møller	12.03 ± 0.01	2.26 ± 0.22	6.11 ± 0.85	74.40 ± 0.19	18.49 ± 0.53	6.63 ± 0.83	74.88 ± 0.30	Pereira et al., 2012
Agrocybe chaxingu Huang	13.7 ± 0.2	5.4 ± 0.0	5.0 ± 0.0	69.3 ± 0.7	25.7	5.0	69.3	Lee et al., 2011
Amanita caesarea (Scop.) Pers.	12.42 ± 0.37	4.88 ± 0.24	53.78 ± 1.22	25.74 ± 1.66	19.16 ± 0.46	54.81 ± 1.20	26.03 ± 1.66	Reis et al., 2011
	14.5 ± 0.5	2.9 ± 0.3	44 ± 1	35 ± 1	19.4 ± 0.5	45.6 ± 0.5	35.0 ± 0.2	Fernandes et al., 2015
Amanita crócea (Quél. in Bourd.) Singer ex Sing	17.66 ± 1.21	3.68 ± 0.23	54.46 ± 0.65	20.14 ± 0.08	24.21 ± 0.59	55.42 ± 0.65	20.37 ± 0.06	Leal et al., 2013
Amanita curtipes E.-J.Gilbert	18.9 ± 0.2	4.1 ± 0.2	54 ± 1	19.2 ± 0.3	25.4 ± 0.3	55.2 ± 0.4	19.4 ± 0.3	Fernandes et al., 2015
Amanita mairei (Foley)	18.08 ± 0.11	3.47 ± 0.02	53.02 ± 0.12	22.02 ± 0.04	23.64 ± 0.15	53.94 ± 0.11	22.42 ± 0.04	Leal et al., 2013
Amanita muscaria (L.:Fr.) Lam.	7.88 ± 0.03	6.13 ± 0.02	60.08 ± 1.03	21.87 ± 1.07	16.46 ± 0.05	61.38 ± 1.02	22.16 ± 1.07	Reis et al., 2011
Amanita umbrinolutea (Secr. ex Gillet)	15.10 ± 0.13	3.87 ± 0.01	58.82 ± 0.08	18.81 ± 0.02	21.18 ± 0.10	59.82 ± 0.12	19.00 ± 0.02	Pereira et al., 2012
Armillaria mellea (Vahl) P. Kumm.	11.04 ± 0.06	3.53 ± 0.01	47.74 ± 0.35	27.71 ± 0.32	17.23 ± 0.07	55.01 ± 0.36	27.76 ± 0.29	Vaz et al., 2011
Boletus armeniacus (Quél.)	15.68 ± 0.34	2.92 ± 0.20	27.61 ± 0.42	48.95 ± 0.06	21.01 ± 0.27	29.67 ± 0.36	49.32 ± 0.09	Pereira et al., 2012
Boletus edulis Bull. Fr.	12.7 ± 0.48	0.67 ± 0.06	8.22 ± 0.34	75.6 ± 0.96	13.4	10.5	76.1	Ayaz et al., 2011
	7.6 ± 0.1	2.89 ± 0.02	34.1 ± 0.2	51.7 ± 0.2	11.6 ± 0.1	35.9 ± 0.2	52.5 ± 0.2	Fernandes et al., 2013a

	11.0 ± 0.1	0.92 ± 0.02	5.7 ± 0.2	77.2 ± 0.1	13.2 ± 0.1	7.4 ± 0.2	79.3 ± 0.1	Fernandes et al., 2014
	7.56 ± 0.05	1.93 ± 0.08	30.41 ± 0.01	57.16 ± 0.01	10.59 ± 0.04	31.90 ± 0.04	57.51 ± 0.00	Heleno et al., 2015c
Boletus erythropus (Pers.)	21.33 ± 1.46	4.15 ± 0.02	14.74 ± 1.19	48.76 ± 0.86	33.62 ± 1.78	16.06 ± 0.99	50.32 ± 0.80	Grangeia et al., 2011
Boletus fragrans (Vittadini)	14.91 ± 0.12	2.39 ± 0.10	19.80 ± 0.81	56.89 ± 0.62	20.75 ± 0.09	21.86 ± 0.71	57.38 ± 0.62	Grangeia et al., 2011
Boletus impolitus Fr.	16.77 ± 0.40	1.10 ± 0.16	14.21 ± 1.45	60.95 ± 1.10	23.19 ± 0.41	15.48 ± 1.42	61.33 ± 1.01	Pereira et al., 2012
Boletus poliporus (Imler ex Bon & G. Moreno)	15.32 ± 0.36	2.93 ± 0.17	34.02 ± 0.06	41.90 ± 0.67	21.54 ± 0.58	36.31 ± 0.06	42.15 ± 0.64	Leal et al., 2013
Boletus regius Krombh.	15.94 ± 0.83	1.63 ± 0.03	21.84 ± 0.45	56.11 ± 0.60	19.50 ± 0.93	23.96 ± 0.30	56.55 ± 0.64	Leal et al., 2013
Bovista aestivalis (Bonord.) Demoulin	21.43 ± 1.70	4.32 ± 0.24	12.63 ± 0.13	41.51 ± 3.75	41.80 ± 2.72	15.53 ± 1.20	42.68 ± 3.92	Pereira et al., 2012
Bovista nigrescens (Pers.)	17.39 ± 0.07	4.19 ± 0.26	21.01 ± 0.24	38.28 ± 0.17	37.78 ± 0.47	23.16 ± 0.26	39.06 ± 0.21	Pereira et al., 2012
Calocybe gambosa (Fr.) Donk	13.57 ± 0.50	3.24 ± 0.12	32.54 ± 1.37	43.88 ± 0.31	21.54 ± 1.62	33.38 ± 1.42	45.07 ± 0.20	Vaz et al., 2011
Calvatia utriformis (Bull.) Jaap.	13.54 ± 0.14	2.43 ± 0.07	6.00 ± 0.13	70.29 ± 0.32	22.37 ± 0.22	6.47 ± 0.11	71.16 ± 0.33	Grangeia et al., 2011
Cantharellus cibarius Fr.	13.7 ± 0.89	3.34 ± 0.19	8.44 ± 0.53	45.6 ± 2.48	17.1	37.3	45.6	Ayaz et al., 2011
Cantharellus tubaeformis (Bull.) Fr.	15.2 ± 0.16	6.19 ± 0.12	57.8 ± 0.39	19.8 ± 0.54	21.7	58.5	19.8	Ayaz et al., 2011
Catathelasma ventricosum (Pk.) Singer	5.12 ± 0.25	2.17 ± 0.25	15.45 ± 0.52	73.92 ± 0.67	7.29 ± 0.45	16.28 ± 0.63	76.42 ± 1.07	Liu et al., 2012a
Chlorophyllum rhacodes (Vittadini) Vellinga	16.35 ± 0.31	1.59 ± 0.03	5.68 ± 0.06	72.61 ± 0.51	20.11 ± 0.35	6.91 ± 0.02	72.98 ± 0.36	Pereira et al., 2012
Chroogomphus fulmineus (R. Heim) Courtec.	12.59 ± 0.38	2.47 ± 0.06	47.09 ± 0.94	31.54 ± 1.47	16.89 ± 0.52	49.77 ± 1.00	33.33 ± 1.52	Reis et al., 2011
Clavariadelphus pistillaris L.: Fr. Donk	16.76 ± 0.81	3.99 ± 0.07	49.11 ± 0.23	24.74 ± 0.82	24.86 ± 0.84	50.11 ± 0.02	25.03 ± 0.86	Pereira et al., 2012
Clavariadelphus truncates (Quél.) Donk	14.80 ± 0.18	2.11 ± 0.01	47.26 ± 0.02	29.77 ± 0.12	21.43 ± 0.06	48.31 ± 0.07	30.26 ± 0.02	Pereira et al., 2012
Clavulina rugosa (Bull.) J. Schröt.	15.2 ± 0.53	5.06 ± 1.04	41.7 ± 1.69	35.5 ± 3.16	20.3	44.3	35.5	Ayaz et al., 2011
Clitocybe costata Kühner & Romagn	12.76 ± 0.07	5.99 ± 0.18	37.27 ± 0.20	34.68 ± 0.92	22.34 ± 0.41	38.02 ± 0.31	39.64 ± 0.87	Pereira et al., 2012
Clitocybe gibba (Pers.) Kumm	13.81 ± 0.16	7.89 ± 0.03	4.91 ± 0.18	64.45 ± 0.15	27.82 ± 0.14	6.16 ± 0.10	66.02 ± 0.24	Pereira et al., 2012

(Continued)

Table 21.2 (Continued)

Mushroom Species	Palmitic Acid (C16:0)	Stearic Acid (C18:0)	Oleic Acid (C18:1n9)	Linoleic Acid (C18:2n6)	Total SFA	Total MUFA	Total PUFA	Reference
Clitocybe maxima (Gartn. ex Mey.: Fr.) Quél	12.64 ± 0.58	5.44 ± 0.42	46.82 ± 1.35	28.34 ± 0.74	18.81 ± 0.59	51.61 ± 1.22	29.58 ± 0.84	Liu et al., 2012a
Clitopilus prunulus (Scop. ex Fr.) P. Kumm	10.11 ± 0.30	2.47 ± 0.11	20.42 ± 0.70	59.92 ± 1.97	16.45 ± 0.84	21.51 ± 0.83	62.04 ± 1.67	Grangeia et al., 2011
Clitocybe odora (Fr.) P. Kumm.	12.46 ± 0.25	3.46 ± 0.26	46.07 ± 0.17	34.90 ± 0.68	18.57 ± 0.55	46.39 ± 0.14	35.04 ± 0.70	Vaz et al., 2011
Clitocybe subconnexa (Murrill)	7.31 ± 0.17	2.15 ± 0.08	42.50 ± 1.19	44.88 ± 0.66	11.00 ± 0.46	43.45 ± 1.23	45.59 ± 0.76	Heleno et al., 2015b
Coprinus comatus (O.F.Müll.) Pers.	10.56 ± 0.44	1.90 ± 0.13	6.27 ± 0.03	74.86 ± 0.95	15.42 ± 0.55	7.12 ± 0.02	77.46 ± 0.57	Vaz et al., 2011
	12.88 ± 0.11	2.27 ± 0.01	13.46 ± 0.06	64.08 ± 0.07	18.72 ± 0.13	15.27 ± 0.06	66.01 ± 0.06	Stojković et al., 2013a
Coprinus comatus (O.F.Müll.) Pers (cultivated)	8.58 ± 0.25	2.09 ± 0.01	36.38 ± 0.17	50.49 ± 0.08	11.82 ± 0.24	36.75 ± 0.13	51.43 ± 0.11	Stojković et al., 2013a
Cortinarius anomalus (Fr.) Fr.	12.12 ± 0.06	2.23 ± 0.02	37.74 ± 0.38	44.39 ± 0.39	15.93 ± 0.04	39.40 ± 0.35	44.68 ± 0.39	Reis et al., 2011
Cortinarius collinitus (Pers.) Fr.	19.03 ± 0.30	2.94 ± 0.10	35.21 ± 0.13	35.89 ± 0.79	24.58 ± 0.58	38.54 ± 0.16	36.87 ± 0.74	Reis et al., 2011
Cortinarius glaucopus (Schaeff)	12.05 ± 0.02	2.98 ± 0.00	24.01 ± 0.85	54.99 ± 1.00	19.60 ± 0.21	25.12 ± 0.80	55.28 ± 1.01	Heleno et al., 2009
Cortinarius praestans Cordier	13.44 ± 0.03	1.78 ± 0.41	20.76 ± 3.04	59.95 ± 3.33	17.93 ± 0.67	21.49 ± 3.09	60.59 ± 3.76	Pereira et al., 2012
Cortinarius violaceus (L.: Fr.) Gray	14.02 ± 0.04	1.67 ± 0.01	14.59 ± 1.26	66.22 ± 1.12	17.46 ± 0.16	15.96 ± 1.30	66.58 ± 1.13	Reis et al., 2011
Craterellus cornucopioides (L.: Fr.) Pers.	10.15 ± 0.06	5.37 ± 0.84	54.02 ± 2.21	28.35 ± 0.97	15.76 ± 0.84	54.45 ± 2.47	29.78 ± 1.03	Liu et al., 2012a
Fistulina hepatica Schaeff.: Fr.	10.42 ± 0.64	2.54 ± 0.12	31.51 ± 0.07	52.37 ± 1.23	15.11 ± 0.96	32.19 ± 0.22	52.70 ± 1.18	Heleno et al., 2009
Flammulina velutipes (Curtis) Singer	10.31 ± 0.39	1.38 ± 0.08	15.08 ± 0.47	56.33 ± 0.14	14.36 ± 0.34	17.56 ± 0.51	68.08 ± 0.17	Pereira et al., 2012
Flammulina velutipes (Curtis) Singer (cultivated)	18.6 ± 0.7	3.1 ± 0.2	5.6 ± 0.1	63.7 ± 1.3	30.7	5.6	63.7	Lee et al., 2011
	11.0 ± 0.2	2.0 ± 0.1	5.7 ± 0.1	45.4 ± 0.1	18.5 ± 0.2	7.2 ± 0.1	74.3 ± 0.2	Reis et al., 2012
Helvella lacunose (Afzel.)	29.05 ± 0.06	5.51 ± 0.03	43.82 ± 0.03	12.21 ± 0.14	39.87 ± 1.16	46.44 ± 0.04	13.70 ± 1.12	Leal et al., 2013
Hericium coralloides coralloides (Scop.) Pers.	23.34 ± 0.66	6.78 ± 0.48	33.74 ± 0.39	30.90 ± 0.20	33.51 ± 0.13	34.59 ± 0.39	31.90 ± 0.22	Heleno et al., 2015a

Hericium erinaceum (Bull.) Persoon	37.57 ± 2.23	7.61 ± 0.72	26.11 ± 1.25	25.10 ± 0.35	47.57 ± 1.47	26.80 ± 1.19	25.63 ± 0.28	Heleno et al., 2015a
Hydnum repandum L., Fr.	10.9 ± 0.10	1.71 ± 0.06	42.4 ± 0.49	42.5 ± 0.63	12.9	44.4	42.7	Ayaz et al., 2011
	13.1 ± 0.1	2.24 ± 0.03	37.2 ± 0.3	39.3 ± 0.3	0.18 ± 0.01	16.6 ± 0.1	43.2 ± 0.2	Fernandes et al., 2013a
Hygrophoropsis aurantiaca (Wulfen) Maire	9.97 ± 0.05	0.92 ± 0.00	17.82 ± 0.00	55.45 ± 0.26	14.75 ± 0.25	20.76 ± 0.03	64.49 ± 0.22	Heleno et al., 2009
Hygrophorus chrysodon (Fr.) Fr.	25.95 ± 0.61	3.88 ± 0.01	57.26 ± 0.57	1.23 ± 0.06	35.32 ± 0.67	63.05 ± 0.55	1.63 ± 0.13	Pereira et al., 2012
Hygrophorus pustulatus (Persoon : Fries) Fries	6.96 ± 0.34	3.96 ± 0.16	51.53 ± 0.05	34.74 ± 0.39	13.18 ± 0.39	51.85 ± 0.01	34.98 ± 0.39	Grangeia et al., 2011
Hypholoma capnoides (Fr.) Quel.	16.43 ± 0.16	4.10 ± 0.07	16.98 ± 0.53	35.67 ± 0.17	44.37 ± 0.58	19.11 ± 0.70	36.53 ± 0.11	Heleno et al., 2009
Ganoderma lucidum (Curtis) P. Karst	12.01 ± 0.01	1.33 ± 0.02	47.24 ± 0.04	33.94 ± 0.03	15.67 ± 0.03	49.63 ± 0.02	34.70 ± 0.01	Stojković et al., 2013b
Ganoderma lucidum (Curtis) P. Karst (cultivated)	18.54 ± 0.13	7.34 ± 0.06	24.06 ± 0.00	39.80 ± 0.05	32.39 ± 0.17	25.19 ± 0.06	42.42 ± 0.11	Stojković et al., 2013b
Gyromitra esculenta (Pers. ex Pers.) Fr.	19.29 ± 0.16	1.64 ± 0.01	13.44 ± 0.06	55.30 ± 0.24	25.76 ± 0.21	17.86 ± 0.01	56.38 ± 0.22	Leal et al., 2013
Laccaria amethystea (Bull. ex Gray) Murr.	6.94 ± 0.44	3.34 ± 0.16	13.74 ± 0.75	74.39 ± 0.24	10.33 ± 0.56	13.74 ± 0.75	75.92 ± 1.33	Liu et al., 2012a
Laccaria laccata (Scop.) Cooke	11.64 ± 0.07	2.02 ± 0.00	60.68 ± 0.01	20.45 ± 0.14	15.44 ± 0.10	63.64 ± 0.06	21.03 ± 0.18	Heleno et al., 2009
	20.0 ± 0.11	1.84 ± 0.04	45.9 ± 0.36	31.6 ± 0.44	21.9	46.6	31.6	Ayaz et al., 2011
Lactarius citriolens Pouzar	5.35 ± 0.01	40.58 ± 0.41	25.00 ± 0.78	22.46 ± 0.03	51.85 ± 0.70	25.42 ± 0.78	22.74 ± 0.08	Vieira et al., 2014
Lactarius salmonicolor R.Heim & Leclair	7.35 ± 0.51	40.13 ± 0.47	18.45 ± 0.04	26.44 ± 0.20	54.00 ± 0.29	18.93 ± 0.04	27.07 ± 0.25	Heleno et al., 2009
Lactarius turpis (Weinm.) Fr.	8.02 ± 0.09	12.60 ± 0.83	26.29 ± 0.98	48.55 ± 0.14	23.73 ± 1.00	27.18 ± 0.98	49.09 ± 0.01	Vieira et al., 2014
Lactarius volemus (Fr.) Fr.	12.19 ± 0.44	6.44 ± 0.59	39.27 ± 0.15	34.38 ± 0.13	23.92 ± 0.18	40.80 ± 0.12	35.28 ± 0.06	Reis et al., 2011
Lentinula edodes (Berk.) Pegler (cultivated)	10.3 ± 0.2	1.6 ± 0.1	2.3 ± 0.3	81.1 ± 0.4	15.1 ± 0.1	2.9 ± 0.3	82.0 ± 0.4	Reis et al., 2012
Lentinus edodes (Berk.) Pegler (cultivated)	9.40 ± 0.11	1.51 ± 0.05	1.87 ± 0.41	82.55 ± 0.58	14.12 ± 0.06	2.62 ± 0.06	83.26 ± 0.52	Heleno et al., 2015c
Lepista inversa (Scop. Fr.) Pat.	16.36 ± 0.23	1.71 ± 0.03	28.78 ± 0.08	44.58 ± 0.08	21.30 ± 0.01	29.14 ± 0.10	49.56 ± 0.10	Heleno et al., 2009

(Continued)

Table 21.2 (Continued)

Mushroom Species	Palmitic Acid (C16:0)	Stearic Acid (C18:0)	Oleic Acid (C18:1n9)	Linoleic Acid (C18:2n6)	Total SFA	Total MUFA	Total PUFA	Reference
Lepista nuda (Bull.) Cooke	11.9 ± 0.19	0.89 ± 0.04	30.8 ± 0.38	53.6 ± 0.59	13	32.7	57.3	Ayaz et al., 2011
Leucoagaricus leucothites Vittad. Wasser	12.16 ± 0.20	1.81 ± 0.11	6.27 ± 0.39	74.72 ± 1.32	18.00 ± 0.84	6.74 ± 0.43	75.25 ± 1.27	Pereira et al., 2012
Leucopaxillus giganteus (Sowerby) Singer	13.9 ± 0.13	3.73 ± 0.05	43.3 ± 0.44	38.3 ± 0.06	18	43.8	38.3	Ayaz et al., 2011
Lyophyllum decastes (Fries: Fries) Singer	10.95 ± 0.04	4.88 ± 0.02	47.43 ± 1.43	32.03 ± 1.39	19.31 ± 0.03	47.94 ± 1.44	32.75 ± 1.41	Grangeia et al., 2011
Lycoperdon echinatum Pers.	10.82 ± 0.35	2.08 ± 0.14	9.16 ± 0.66	69.92 ± 0.71	19.45 ± 0.11	9.75 ± 0.56	70.80 ± 0.67	Grangeia et al., 2011
Lycoperdon umbrinum Pers.	19.92 ± 0.12	7.14 ± 0.5	22.83 ± 0.33	29.36 ± 0.11	42.48 ± 0.49	24.79 ± 0.77	32.74 ± 0.19	Pereira et al., 2012
Macrolepiota excoriate (Schaeff.) M.M. Moser	17.83 ± 0.05	1.94 ± 0.01	10.21 ± 0.36	66.19 ± 0.05	22.49 ± 0.29	11.10 ± 0.37	66.41 ± 0.08	Grangeia et al., 2011
Macrolepiota procera (Scop.) Singer	21.4 ± 0.2	1.77 ± 0.03	7.6 ± 0.1	65.0 ± 0.2	25.2 ± 0.2	9.3 ± 0.1	65.5 ± 0.2	Fernandes et al., 2013b
Morchella esculenta (L.) Pers.	9.54 ± 0.04	2.63 ± 0.01	12.43 ± 0.26	71.81 ± 0.16	13.73 ± 0.21	13.82 ± 0.18	72.45 ± 0.03	Heleno et al., 2013a
Pleurotus eryngii (DC.) Quél.	17.44 ± 0.21	4.77 ± 0.08	47.52 ± 0.07	24.71 ± 0.28	25.79 ± 0.18	49.05 ± 0.24	25.17 ± 0.24	Reis et al., 2014
Pleurotus eryngii (DC.) Quél. (cultivated)	12.8 ± 0.8	1.7 ± 0.3	12.3 ± 0.6	68.8 ± 2.1	17.4 ± 1.1	13.1 ± 1.0	69.4 ± 2.1	Reis et al., 2012
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm.	11.0 ± 0.3	2.6 ± 0.2	4.8 ± 0.2	76.5 ± 0.6	17.4	6.1	76.5	Lee et al., 2011
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm. (cultivated)	11.2 ± 0.2	1.6 ± 0.2	12.3 ± 0.3	68.9 ± 0.8	17.0 ± 0.5	13.6 ± 0.3	69.4 ± 0.7	Reis et al., 2012
Ramaria aurea (Schaeff.) Quél.	7.32 ± 0.04	4.07 ± 0.09	56.92 ± 0.49	25.60 ± 0.17	15.27 ± 0.23	58.47 ± 0.40	26.26 ± 0.17	Pereira et al., 2012
Russula aurea Pers.	9.28 ± 0.14	5.29 ± 1.24	40.63 ± 2.42	40.32 ± 3.31	17.25 ± 1.02	42.09 ± 2.45	40.66 ± 3.46	Leal et al., 2013
Russula cyanoxantha (Schaeff.) Fr.	12.95 ± 0.22	11.10 ± 0.15	28.39 ± 1.11	43.65 ± 1.45	26.90 ± 0.36	29.06 ± 1.06	44.04 ± 1.43	Grangeia et al., 2011
Russula delica Fr.	12.02 ± 0.01	10.34 ± 0.10	41.20 ± 0.06	27.15 ± 0.05	27.20 ± 0.06	44.89 ± 0.00	27.91 ± 0.06	Heleno et al., 2009
	12.2 ± 0.1	1.50 ± 0.01	16.3 ± 0.1	67.5 ± 0.1	15.0 ± 0.1	17.2 ± 0.1	679 ± 0.1	Fernandes et al., 2014

Russula olivacea (Schaeff.) Fr.	16.13 ± 0.39	2.78 ± 0.03	25.99 ± 0.12	50.20 ± 0.28	21.75 ± 0.39	27.40 ± 0.07	50.85 ± 0.32	Grangeia et al., 2011
Russula rosea Pers.	9.07 ± 0.13	34.6 ± 0.26	31.5 ± 0.31	23.2 ± 0.47	44.3	38.1	36.2	Ayaz et al., 2011
Russula sardonia Fr.	7.84 ± 0.47	6.56 ± 0.25	63.19 ± 1.72	16.93 ± 1.25	15.71 ± 0.29	65.12 ± 1.62	19.17 ± 1.33	Reis et al., 2011
Russula virescens (Schaeff.) Fr.	17.31 ± 0.19	7.16 ± 0.12	40.27 ± 0.04	29.18 ± 0.04	28.78 ± 0.08	41.51 ± 0.01	29.71 ± 0.09	Leal et al., 2013
Suillus luteus (L.: Fries) Gray	10.57 ± 0.05	2.06 ± 0.07	31.24 ± 0.26	52.31 ± 0.68	14.32 ± 0.19	32.93 ± 0.45	52.75 ± 0.64	Reis et al., 2011
Stropharia rugoso-annulata Farlow apud Murr.	9.46 ± 0.64	3.95 ± 0.53	39.32 ± 1.04	45.42 ± 0.75	13.56 ± 0.43	39.99 ± 1.42	46.45 ± 1.17	Liu et al., 2012a
Suillus mediterraneensis (Jacquetant & Blum) Redeuilh	11.93 ± 0.08	3.56 ± 0.01	36.42 ± 0.03	43.72 ± 0.17	18.04 ± 0.11	37.92 ± 0.06	44.03 ± 0.17	Heleno et al., 2009
Suillus variegates (Sw.) Kuntze	12.71 ± 0.29	3.47 ± 0.08	42.00 ± 0.26	37.44 ± 0.13	18.09 ± 0.29	44.24 ± 0.16	37.67 ± 0.12	Pereira et al., 2012
Tricholoma imbricatum (Fr.) P.Kumm	7.44 ± 0.15	4.10 ± 0.01	51.53 ± 0.42	33.03 ± 0.14	14.91 ± 0.27	51.80 ± 0.41	33.29 ± 0.14	Heleno et al., 2009
Tricholoma saponaceum (Fr.) P.Kumm.	12.6 ± 0.23	2.96 ± 0.31	38.8 ± 1.24	43.1 ± 1.81	16.0	40.9	43.1	Ayaz et al., 2011
Tricholoma ustale (Fr.) P. Kumm	9.72 ± 0.21	3.46 ± 0.21	23.46 ± 0.57	59.29 ± 0.35	16.22 ± 0.28	24.37 ± 0.64	59.41 ± 0.35	Reis et al., 2011
Xerocomus badius (Fr.) E.-J.Gilbert	15.47 ± 0.12	1.96 ± 0.15	38.15 ± 0.01	42.22 ± 0.11	18.52 ± 0.10	39.03 ± 0.03	42.46 ± 0.01	Heleno et al., 2015c
Volvopluteus gloiocephalus (DC.) Vizzini, Contu & Justo)	7.42 ± 0.17	2.09 ± 0.07	25.96 ± 0.02	59.33 ± 0.15	12.56 ± 0.17	26.47 ± 0.01	60.97 ± 0.17	Heleno et al., 2015b

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.

Table 21.3 Main sugars (g/100 g dw) found in wild and cultivated mushroom species.

Mushroom Species	Mannitol	Trehalose	Total Sugars	Reference
Agaricus albertii (Bon.)	4.78 ± 0.02	0.70 ± 0.04	5.98 ± 0.03	Reis et al., 2014
Agaricus bisporus (J.E. Lange) Imbach (white, cultivated)	64.15 ± 2.18	1.83 ± 0.23	66.32 ± 2.41	Reis et al., 2012
Agaricus bisporus (J.E. Lange) Imbach (brown, cultivated)	47.97 ± 2.39	2.63 ± 0.36	51.08 ± 2.39	Reis et al., 2012
Agaricus campestris (L.)	16.34 ± 0.78	0.72 ± 0.07	97.88 ± 0.45	Beluhan et al., 2011
	16.94 ± 2.71	3.62 ± 0.33	20.56 ± 3.03	Pereira et al., 2012
Agaricus comtulus Fries	15.39 ± 0.73	3.60 ± 0.06	18.99 ± 0.78	Pereira et al., 2012
Agaricus excellens (F.H. Møller) Nauta	0.77 ± 0.01	0.14 ± 0.02	1.51 ± 0.01	Reis et al., 2014
Agaricus lutosus (Møller) Møller	16.42 ± 0.62	3.35 ± 0.19	19.77 ± 0.43	Pereira et al., 2012
Amanita caesarea (Scop.) Pers.	2.10 ± 0.09	3.15 ± 0.01	5.24 ± 0.08	Reis et al., 2011
	0.30 ± 0.02	0.58 ± 0.03	0.88 ± 0.03	Fernandes et al., 2015
Amanita crócea (Quél. in Bourd.) Singer ex Singer	3.57 ± 0.33	4.54 ± 0.37	8.11 ± 0.69	Leal et al., 2013
Amanita curtipes E.-J.Gilbert	3.9 ± 0.1	8.9 ± 0.2	15.1 ± 0.2	Fernandes et al., 2015
Amanita mairei (Foley)	1.47 ± 0.05	1.31 ± 0.05	2.78 ± 0.00	Leal et al., 2013
Amanita muscaria (L.:Fr.) Lam.	1.02 ± 0.02	0.94 ± 0.03	1.97 ± 0.04	Reis et al., 2011
Amanita pantherina (DC. ex Fr.) Krombh.	4.41 ± 0.10	5.31 ± 0.33	9.74 ± 0.43	Reis et al., 2011
Amanita umbrinolutea (Secr. ex Gillet)	31.83 ± 0.69	10.06 ± 0.58	41.89 ± 1.27	Pereira et al., 2012
Armillaria mellea (Vahl) P. Kumm.	5.45 ± 0.04	9.33 ± 0.04	15.66 ± 0.04	Vaz et al., 2011
Bovista aestivalis (Bonord.) Demoulin	nd	0.38 ± 0.08	0.38 ± 0.08	Pereira et al., 2012
Bovista nigrescens (Pers.)	0.93 ± 0.01	5.09 ± 0.29	6.02 ± 0.30	Pereira et al., 2012
Boletus armeniacus (Quél.)	23.56 ± 2.43	5.62 ± 0.35	39.64 ± 1.17	Pereira et al., 2012
Boletus edulis Bull. Fr.	3.72 ± 0.21	9.92 ± 0.04	59.89 ± 0.13	Beluhan et al., 2011
	1.13 ± 0.03	17.7 ± 0.1	20.9 ± 0.1	Fernandes et al., 2013a
	0.15 ± 0.02	3.2 ± 0.1	4.0 ± 0.1	Fernandes et al., 2014
	3.14 ± 1.18	9.29 ± 0.51	14.38 ± 2.47	Heleno et al., 2015c
Boletus erythropus (Pers.)	27.90 ± 0.30	4.84 ± 0.51	34.46 ± 0.24	Grangeia et al., 2011
Boletus fragrans (Vittadini)	36.97 ± 0.33	3.14 ± 0.26	42.37 ± 0.60	Grangeia et al., 2011
Boletus impolitus Fr.	8.08 ± 0.08	1.84 ± 0.05	10.23 ± 0.02	Pereira et al., 2012
Boletus poliporus (Imler ex Bon & G. Moreno)	19.33 ± 0.90	1.99 ± 0.01	41.26 ± 1.00	Leal et al., 2013
Boletus regius Krombh.	6.25 ± 0.35	0.66 ± 0.03	20.95 ± 1.04	Leal et al., 2013
Calocybe gambosa (Fr.) Donk	0.34 ± 0.09	7.69 ± 0.24	36.39 ± 0.18	Beluhan et al., 2011
	0.29 ± 0.01	7.96 ± 0.28	8.26 ± 0.29	Vaz et al., 2011
Calvatia utriformis (Bull.) Jaap.	nd	0.40 ± 0.01	0.40 ± 0.01	Grangeia et al., 2011

Table 21.3 (Continued)

Mushroom Species	Mannitol	Trehalose	Total Sugars	Reference
Cantharellus cibarius Fr.	8.56 ± 0.02	6.68 ± 0.09	48.78 ± 0.12	Beluhan et al., 2011
Chlorophyllum rhacodes (Vittadini) Vellinga	18.43 ± 0.45	25.57 ± 0.38	44.00 ± 0.07	Pereira et al., 2012
Chroogomphus fulmineus (R. Heim) Courtec.	7.21 ± 1.18	5.61 ± 0.84	16.11 ± 2.67	Reis et al., 2011
Clavariadelphus pistillaris L.:Fr. Donk	24.43 ± 3.25	nd	25.36 ± 3.47	Pereira et al., 2012
Clavariadelphus truncatus (Quél.) Donk	43.34 ± 2.76	nd	43.74 ± 2.79	Pereira et al., 2012
Clitocybe costata Kühner & Romagn	15.53 ± 0.85	10.99 ± 0.54	26.65 ± 1.40	Pereira et al., 2012
Clitocybe gibba (Pers.) Kumm	0.63 ± 0.02	1.04 ± 0.11	1.67 ± 0.08	Pereira et al., 2012
Clitocybe odora (Fr.) P. Kumm.	0.59 ± 0.02	7.77 ± 0.30	8.36 ± 0.32	Vaz et al., 2011
Clitocybe subconnexa (Murrill)	24.71 ± 0.55	6.00 ± 0.01	30.71 ± 0.56	Heleno et al., 2015b
Clitopilus prunulus (Scop. ex Fr.) P. Kumm	0.95 ± 0.10	0.95 ± 0.16	1.90 ± 0.27	Grangeia et al., 2011
Coprinus comatus (O.F.Müll.) Pers.	0.40 ± 0.04	42.82 ± 2.59	43.23 ± 2.62	Vaz et al., 2011
	1.84 ± 0.14	5.41 ± 0.38	7.25 ± 0.52	Stojković et al., 2013a
Coprinus comatus (O.F.Müll.) Pers (cultivated)	1.41 ± 0.11	8.75 ± 0.02	10.27 ± 0.12	Stojković et al., 2013a
Cortinarius anomalus (Fr.) Fr.	1.85 ± 0.03	9.92 ± 0.11	11.78 ± 0.14	Reis et al., 2011
Cortinarius collinitus (Pers.) Fr.	8.12 ± 0.12	2.82 ± 0.06	10.95 ± 0.06	Reis et al., 2011
Cortinarius glaucopus (Schaeff)	1.06 ± 0.16	18.66 ± 0.37	19.72 ± 0.35	Heleno et al., 2009
Cortinarius praestans Cordier	0.37 ± 0.01	60.51 ± 2.31	60.88 ± 2.30	Pereira et al., 2012
Cortinarius violaceus (L.: Fr.) Gray	6.35 ± 0.04	7.90 ± 0.22	14.26 ± 0.18	Reis et al., 2011
Craterellus cornucopioides (Scop.) Pers.	11.21 ± 0.12	0.09 ± 0.05	14.08 ± 0.32	Beluhan et al., 2011
Entoloma clypeatum (L.) P. Kumm.	6.67 ± 0.07	3.05 ± 0.42	21.50 ± 0.37	Beluhan et al., 2011
Fistulina hepatica Schaeff.: Fr.	2.12 ± 0.22	2.95 ± 0.22	12.82 ± 0.93	Heleno et al., 2009
Flammulina velutipes (Curtis) Singer	7.90 ± 0.15	2.97 ± 0.67	30.10 ± 0.59	Beluhan et al., 2011
	5.98 ± 1.19	15.08 ± 1.60	24.61 ± 3.05	Pereira et al., 2012
Flammulina velutipes (Curtis) Singer (cultivated)	7.80 ± 0.91	21.68 ± 1.82	68.34 ± 5.61	Reis et al., 2012
Ganoderma lucidum (Curtis) P. Karst	1.60 ± 0.08	0.38 ± 0.00	9.14 ± 0.14	Stojković et al., 2013b
Ganoderma lucidum (Curtis) P. Karst (cultivated)	0.45 ± 0.04	0.30 ± 0.06	0.75 ± 0.03	Stojković et al., 2013b
Gyromitra esculenta (Pers. ex Pers.) Fr.	4.17 ± 0.21	1.96 ± 0.04	6.13 ± 0.24	Leal et al., 2013
Helvella lacunosa (Afzel.)	4.13 ± 0.69	0.23 ± 0.07	4.36 ± 0.62	Leal et al., 2013
Hericium coraloides coralloides (Scop.) Pers.	3.86 ± 0.17	0.68 ± 0.03	10.79 ± 0.51	Heleno et al., 2015a
Hericium erinaceum (Bull.) Persoon	5.63 ± 0.22	0.54 ± 0.07	23.63 ± 0.94	Heleno et al., 2015a
Hydnum repandum L., Fr.	13.0 ± 0.2	4.4 ± 0.1	17.4 ± 0.2	Fernandes et al., 2013a

(Continued)

Table 21.3 (Continued)

Mushroom Species	Mannitol	Trehalose	Total Sugars	Reference
Hygrophoropsis aurantiaca (Wulfen) Maire	4.31 ± 0.68	7.56 ± 1.01	13.40 ± 0.96	Heleno et al., 2009
Hygrophorus chrysodon (Fr.) Fr.	nd	7.27 ± 1.40	7.27 ± 1.40	Pereira et al., 2012
Hygrophorus pustulatus (Persoon : Fries) Fries	35.37 ± 5.24	3.00 ± 0.01	38.52 ± 5.29	Grangeia et al., 2011
Hypholoma capnoides (Fr.) Quel.	0.38 ± 0.04	1.58 ± 0.40	1.96 ± 0.44	Heleno et al., 2009
Laccaria laccata (Scop.) Cooke	0.64 ± 0.05	5.81 ± 0.33	6.45 ± 0.34	Heleno et al., 2009
Lactarius citriolens Pouzar	8.31 ± 0.30	0.45 ± 0.01	8.76 ± 0.29	Vieira et al., 2014
Lactarius quietus (Fr. ex Fr.) Fr.	9.84 ± 0.30	4.73 ± 0.01	14.57 ± 0.31	Reis et al., 2011
Lactarius salmonicolor R.Heim & Leclair	13.48 ± 1.95	0.35 ± 0.05	13.83 ± 1.98	Heleno et al., 2009
Lactarius turpis (Weinm.) Fr.	19.21 ± 0.45	0.33 ± 0.03	19.54 ± 0.47	Vieira et al., 2014
Lactarius volemus (Fr.) Fr.	27.29 ± 1.81	0.93 ± 0.00	28.22 ± 1.81	Reis et al., 2011
Lentinula edodes (Berk.) Pegler (cultivated)	49.51 ± 6.43	16.72 ± 1.83	69.64 ± 6.43	Reis et al., 2012
Lentinus edodes (Berk.) Pegler (cultivated)	5.16 ± 0.21	8.41 ± 0.24	15.61 ± 0.59	Heleno et al., 2015c
Lepista inversa (Scop. Fr.) Pat.	1.86 ± 0.08	4.32 ± 0.27	6.18 ± 0.35	Heleno et al., 2009
Leucoagaricus leucothites Vittad. Wasser	13.33 ± 2.77	3.21 ± 0.70	16.54 ± 3.47	Pereira et al., 2012
Lyophyllum decastes (Fries: Fries) Singer	11.03 ± 0.74	3.98 ± 0.54	15.01 ± 1.28	Grangeia et al., 2011
Lycoperdon echinatum Pers.	0.85 ± 0.03	1.38 ± 0.09	2.23 ± 0.06	Grangeia et al., 2011
Lycoperdon umbrinum Pers.	0.28 ± 0.04	0.18 ± 0.11	1.46 ± 0.07	Pereira et al., 2012
Macrolepiota excoriate (Schaeff.) M.M. Moser	4.10 ± 0.04	2.75 ± 0.04	6.85 ± 0.09	Grangeia et al., 2011
Macrolepiota procera (Scop.) Singer	2.42 ± 0.18	0.11 ± 0.02	24.40 ± 0.09	Beluhan et al., 2011
	5.2 ± 0.1	9.1 ± 0.3	15.7 ± 0.4	Fernandes et al., 2013b
Morchella elata Fr.	5.43 ± 0.21	1.09 ± 0.02	59.13 ± 0.12	Beluhan et al., 2011
Morchella esculenta (L.) Pers.	11.54 ± 0.18	3.41 ± 0.01	15.66 ± 0.13	Heleno et al., 2013a
Pleurotus eryngii (DC.) Quél.	1.40 ± 0.09	14.21 ± 0.23	15.63 ± 0.31	Reis et al., 2014
Pleurotus eryngii (DC.) Quél. (cultivated)	5.45 ± 0.09	72.82 ± 1.72	78.81 ± 1.73	Reis et al., 2012
Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm.	9.82 ± 0.55	1.79 ± 0.12	37.75 ± 0.39	Beluhan et al., 2011
Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm. (cultivated)	4.99 ± 0.37	40.81 ± 0.28	45.89 ± 0.18	Reis et al., 2012
Ramaria aurea (Schaeff.) Quél.	15.11 ± 0.30	0.95 ± 0.11	17.59 ± 0.21	Pereira et al., 2012
Russula aurea Pers.	9.56 ± 0.14	2.29 ± 0.33	11.85 ± 0.19	Leal et al., 2013
Russula cyanoxantha (Schaeff.) Fr.	16.18 ± 0.61	1.64 ± 0.32	18.16 ± 0.26	Grangeia et al., 2011
Russula delica Fr.	18.41 ± 0.38	0.21 ± 0.03	18.62 ± 0.35	Heleno et al., 2009
	4.28 ± 0.02	2.83 ± 0.03	10.3 ± 0.2	Fernandes et al., 2014
Russula olivacea (Schaeff.) Fr.	15.25 ± 0.24	0.71 ± 0.07	16.19 ± 0.34	Grangeia et al., 2011
Russula sardonia Fr.	20.14 ± 0.32	nd	20.14 ± 0.32	Reis et al., 2011
Russula virescens (Schaeff.) Fr.	10.90 ± 0.13	0.20 ± 0.01	11.10 ± 0.13	Leal et al., 2013
Suillus luteus (L.: Fries) Gray	1.29 ± 0.03	1.35 ± 0.04	2.64 ± 0.07	Reis et al., 2011

Table 21.3 (Continued)

Mushroom Species	Mannitol	Trehalose	Total Sugars	Reference
Suillus mediterraneensis (Jacquetant & Blum) Redeuilh	2.89 ± 0.31	1.18 ± 0.19	8.10 ± 1.11	Heleno et al., 2009
Suillus variegates (Sw.) Kuntze	nd	4.85 ± 0.28	4.85 ± 0.28	Pereira et al., 2012
Tricholoma imbricatum (Fr.) P.Kumm.	10.53 ± 0.28	6.56 ± 0.22	17.09 ± 0.48	Heleno et al., 2009
Tricholoma ustale (Fr.) P. Kumm	12.53 ± 0.51	1.67 ± 0.07	14.20 ± 0.44	Reis et al., 2011
Xerocomus badius (Fr.) E.-J.Gilbert	6.20 ± 0.25	4.62 ± 0.02	11.77 ± 0.03	Heleno et al., 20015c
Volvopluteus gloiocephalus (DC.) Vizzini, Contu & Justo)	1.45 ± 0.10	1.92 ± 0.11	3.37 ± 0.21	Heleno et al., 20015b

nd, not detected; dw, dry weight.
Other sugars identified in low amounts – **Arabinose**: *A. mellea* (Vaz et al., 2011), *C. fulmineus* (Reis et al., 2011), *F. hepatica* (Heleno et al., 2009), *H. coralloides* (Heleno et al., 2015a), *H. erinaceum* (Heleno et al., 2015a), *H. aurantiaca* (Heleno et al., 2009) and *S. mediterraneensis* (Heleno et al., 2009). **Fructose:** *A. albertii* (Reis et al., 2014), *A. bisporus* white, cultivated (Reis et al., 2012), *A. bisporus* brown, cultivated (Reis et al., 2012), *A. excellens* (Reis et al., 2014), *A. curtipes* (Fernandes et al., 2015), *B. armeniacus* (Pereira et al., 2012), *B.edulis* (Fernandes et al., 2013; Fernandes et al., 2014; Heleno et al., 2015c), *B. erythropus* (Grangeia et al., 2011), *B. fragrans* (Grangeia et al., 2011), *B. impolitus* (Pereira et al., 2012), *B. poliporus* (Leal et al., 2013), *B. regius* (Leal et al., 2013), *C. pistillaris* (Pereira et al., 2012), *C. truncatus* (Pereira et al., 2012), *C. subconnexa* (Heleno et al., 2015b), *C. comatus* cultivated (Stojković et al., 2013a), *F. velutipes* cultivated (Reis et al., 2012), *G. lucidum* (Stojković et al., 2013b), *H. pustulatus* (Grangeia et al., 2011), *L. edodes* cultivated (Reis et al., 2012), *L. edodes* cultivated (Heleno et al., 2015c), *M. procera* (Fernandes et al., 2013b), *M. esculenta* (Heleno et al., 2013a), *P. eryngii* cultivated (Reis et al., 2012), *P. ostreatus* cultivated (Reis et al., 2012), *R. cyanoxantha* (Pereira et al., 2012), *R. olivacea* (Grangeia et al., 2011) and *X. badius* (Heleno et al., 20015c). **Mannose**: *A. campestris*, *B. edulis*, *C. gambosa*, *C. cibarius*, *F. velutipes*, *M. procera*, *M. elata* and *P. ostreatus* (Beluhan et al., 2011). **Glucose**: *A. campestris* (Beluhan et al., 2011), *B. edulis* (Beluhan et al., 2011; Fernandes et al., 2013a), *C. gambosa*, (Beluhan et al., 2011), *C. cibarius* (Beluhan et al., 2011), *C. cornucopioides* (Beluhan et al., 2011), *E. clypeatum* (Beluhan et al., 2011), *F. velutipes* (Beluhan et al., 2011), *G. lucidum* (Beluhan et al., 2011), *M. procera* (Stojković et al., 2013b), *M. elata* (Beluhan et al., 2011), *P. ostreatus* (Beluhan et al., 2011), and *R. delica* (Fernandes et al., 2014). **Sucrose**: *B. edulis* (Fernandes et al., 2014), *F. velutipes* cultivated (Reis et al., 2012), *G. lucidum* (Stojković et al., 2013b), *P. eryngii* cultivated (Reis et al., 2012), and *R. delica* (Fernandes et al., 2014). **Melezitose**: *M. procera* (Fernandes et al., 2013b).

Wild-grown mushrooms are able to accumulate in their fruiting bodies large amounts of both macro- and micro-elements that are essential to fungi and its consumers (Wang et al., 2014). The mineral element contents in different mushroom species are given in Table 21.4. Potassium (K) and phosphorus (P) are two prevailing elements in fruiting bodies and are usually followed by Ca, Mg, Na, and Fe. Potassium content ranges between 1300 μg/g dw in *Macrocybe gigantea* (Massee) Pegler et Lodge (Liu et al., 2012b) and 46926 μg/g dw in *Volvopluteus gloiocephalus* (DC.) Vizzini, Contu, and Justo (Heleno et al., 2015b). Phosphorus content ranges between 1005 μg/g dw in *Lentinus cladopus* Leveille (Mallikarjuna et al., 2012) and 7290 μg/g dw in *Stropharia rugoso-annulata* Farlow apud Murr. (Liu et al., 2012a).

For elements such as Zn, Cu, and Mn, a large variation of values was noted, ranging from 16–140, 10–87, and 1–83 μg/g dw, respectively (Table 21.4). Mushrooms can also have low contents of toxic elements, such as arsenic (As), cadmium (Cd), and lead (Pb), which were described in *Clitocybe maxima* (Gartn. ex Mey.: Fr.) Quél and *Laccaria amethystea* (Bull. ex Gray) Murr. (Liu et al., 2012a): 0.05 and 0.72 μg/g dw, respectively. Cd was found in some species, and ranges between 0.07 μg/g dw in *Armillaria tabescens* (Scop.) Emel and 1.80 μg/g dw in *F. hepatica* (Ouzouni et al., 2009). Pb ranges from 0.07 μg/g dw in *Stropharia rugoso-annulata* Farlow apud Murr and 1.44 μg/g dw in *C. maxima* (Liu et al., 2012a). These toxic elements were

Table 21.4 Trace metals (µg/g dw) found in wild and cultivated mushroom species.

Mushroom Species	Magnesium (Mg)	Copper (Cu)	Manganese (Mn)	Iron (Fe)	Zinc (Zn)	Lead (Pb)	Cadmium (Cd)	Nickel (Ni)	References
Amanita caesarea (Scop.) Pers.	833.1 ± 4.94	19.32 ± 0.21	47.99 ± 0.78	356.9 ± 4.64	65.65 ± 0.48	0.09 ± 0.02	1.30 ± 0.06	0.76 ± 0.02	Ouzouni et al., 2009
Armillaria mellea (Vahl) P. Kumm	1063.1 ± 7.71	17.38 ± 0.34	55.59 ± 1.59	499.0 ± 7.82	54.12 ± 0.92	0.49 ± 0.03	1.67 ± 0.03	2.58 ± 0.05	Ouzouni et al., 2009
Armillaria tabescens (Scop.) Emel	1150.7 ± 41.45	17.47 ± 0.43	11.18 ± 0.27	60.40 ± 3.45	64.45 ± 0.68	0.79 ± 0.03	1.80 ± 0.14	4.94 ± 0.11	Ouzouni et al., 2009
Boletus aureus Schaeff.	755.1 ± 7.33	41.47 ± 1.67	18.31 ± 0.46	112.8 ± 5.80	89.45 ± 2.21	0.09 ± 0.02	0.23 ± 0.02	1.61 ± 0.04	Ouzouni et al., 2009
Boletus griseus Forst	200 ± 72	52 ± 11	63 ± 17	47 ± 18	94 ± 36	nd	nd	nd	Liu et al., 2012b
Boletus speciosus Forst	110 ± 72	28 ± 10	2.0 ± 0.8	78 ± 34	50 ± 26	nd	nd	nd	Liu et al., 2012b
Cantharellus cibarius Fr.	866.3 ± 11.9	32.49 ± 0.30	22.09 ± 0.54	118.2 ± 2.24	54.29 ± 1.23	nd	0.38 ± 0.10	1.07 ± 0.03	Ouzouni et al., 2009
Catathelasma ventricosum (Pk.) Singer	1538 ± 352	38 ± 4	9 ± 2.31	673 ± 22	88 ± 12	nd	0.58 ± 0.35	nd	Liu et al., 2012a
Clitocybe maxima (Gartn. ex Mey.: Fr.) Quél	520 ± 132	52 ± 12	33 ± 6	308 ± 10	127 ± 24	1.44 ± 0.42	0.29 ± 0.14	nd	Liu et al., 2012a
Clitocybe subconnexa (Murrill)	1489 ± 13.6	52.2 ± 0.5	1.0 ± 0.1	65.3 ± 1.8	63.7 ± 6.4	nd	nd	nd	Heleno et al., 2015b
Craterellus cornucopioides (L.: Fr.) Pers.	978 ± 35	43 ± 11	27 ± 6	413 ± 18	61 ± 11	nd	2.07 ± 0.22	nd	Liu et al., 2012a
Fistulina hepatica Schaeff.: Fr.	898.3 ± 9.38	7.38 ± 0.05	7.19 ± 0.04	38.90 ± 1.97	34.43 ± 0.47	0.14 ± 0.03	0.07 ± 0.01	1.74 ± 0.05	Ouzouni et al., 2009
Hericium coraloides (Scop.) Pers.	1340 ± 38	7.2 ± 0.7	3.1 ± 0.3	779.6 ± 36.4	47.6 ± 4.8	nd	nd	nd	Heleno et al., 2015a
Hericium erinaceum (Bull.) Persoon	856 ± 34.9	2.2 ± 0.2	0.9 ± 0.1	67.7 ± 0.6	21.1 ± 1.2	nd	nd	nd	Heleno et al., 2015a
Hygrophorus russula (Schaeff.: Fr.) Quélet.	758.4 ± 8.29	9.44 ± 0.07	34.14 ± 0.90	300.7 ± 3.87	57.01 ± 0.87	0.08 ± 0.01	1.17 ± 0.04	0.86 ± 0.03	Ouzouni et al., 2009
Laccaria amethystea (Bull. ex Gray) Murr.	1482 ± 241	36 ± 7	35 ± 3	211 ± 19	59 ± 35	0.74 ± 0.22	0.62 ± 0.07	nd	Liu et al., 2012a
Lactarius hygrophoroides Berk. et M.A. Curtis	140 ± 65	28 ± 14	3.7 ± 0.8	28 ± 13	16 ± 6	nd	nd	nd	Liu et al., 2012b
Lentinula edodes (Berk.) Pegler (cultivated)	407 ± 12	14.8 ± 0.3	10 ± 3.2	148 ± 23	94.4 ± 2.4	nd	nd	nd	Mallikarjuna et al., 2012
Lentinus cladopus Leveille	211 ± 35	9.7 ± 0.1	5.4 ± 0.1	353 ± 35.5	15.8 ± 2.1	nd	nd	nd	Mallikarjuna et al., 2012
Lepista nuda (Bull.) Cooke	949.8 ± 13.38	75.06 ± 1.55	33.65 ± 0.83	74.6 ± 0.63	98.99 ± 1.10	1.16 ± 0.04	0.25 ± 0.04	1.39 ± 0.03	Ouzouni et al., 2009

Leucopaxillus giganteus (Sowerby) Singer	84 ± 15	50 ± 20	60 ± 20	510 ± 250	85 ± 12	nd	nd	nd	Liu et al., 2012b
Macrocybe gigantea (Massee) Pegler et Lodge	550 ± 170	13 ± 7	5.9 ± 2.5	79 ± 23	160 ± 49	nd	nd	nd	Liu et al., 2012b
Melanoleuca arcuata (Bull.) Singer	230 ± 100	22 ± 10	1.4 ± 1.0	22 ± 13	38 ± 20	nd	nd	nd	Liu et al., 2012b
Morchella deliciosa Fr.	130 ± 35	55 ± 23	70 ± 22	42 ± 18	58 ± 36	nd	nd	nd	Liu et al., 2012b
Mycena haematopus (Pers.) P. Kumm.	270 ± 130	23 ± 5	24 ± 7	180 ± 43	54 ± 27	nd	nd	nd	Liu et al., 2012b
Pleurotus djamor Sacc	316 ± 17	14.5 ± 0.8	11.2 ± 0.3	148 ± 9.1	92.1 ± 0.3	nd	nd	nd	Mallikarjuna et al., 2012
Pleurotus florida (Block & Tsao)	359 ± 19	10.6 ± 0.6	6.2 ± 0.3	62.7 ± 4.1	50.6 ± 0.4	nd	nd	nd	Mallikarjuna et al., 2012
Pulveroboletus ravenelii (Berk. et M.A. Curtis) Murrill	210 ± 70	58 ± 16	58 ± 20	370 ± 133	34 ± 8	nd	nd	nd	Liu et al., 2012b
Ramaria largentii Marr & D.E. Stuntz	837.5 ± 5.03	17.79 ± 0.23	62.63 ± 0.44	302.1 ± 5.48	46.33 ± 1.08	0.12 ± 0.02	1.13 ± 0.03	9.93 ± 0.10	Ouzouni et al., 2009
Russula delica Fr.	688.7 ± 5.25	51.71 ± 0.30	16.61 ± 0.32	81.80 ± 1.26	56.58 ± 0.54	nd	0.22 ± 0.03	1.90 ± 0.02	Ouzouni et al., 2009
Stropharia rugoso-annulata Farlow apud Murr.	1135 ± 421	29 ± 11	59 ± 11	195 ± 38	102 ± 37	0.07 ± 0.02	1.17 ± 0.32	nd	Liu et al., 2012a
Tricholoma matsutake (S. Itô et S. Imai) Singer	370 ± 74	20 ± 7	3.0 ± 0.9	34 ± 24	62 ± 28	nd	nd	nd	Liu et al., 2012b
	nd	87.2 ± 0.6	83.1 ± 0.2	369 ± 0.3	140 ± 0.7	nd	nd	nd	Liu et al., 2010
Volvopluteus gloiocephalus (DC.) Vizzini, Contu & Justo)	2001.9 ± 145.7	50.1 ± 4.4	1.3 ± 0.1	699.1 ± 68.9	108.9 ± 1.2	nd	nd	nd	Heleno et al., 2015b

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Species	**Cobalt (Co)**	**Chromium (Cr)**	**Selenium (Se)**	**Arsenic (As)**	**Phosphorus (P)**	**Sodium (Na)**	**Potassium (K)**	**Calcium (Ca)**	**References**
Amanita caesarea (Scop.) Pers.	0.75 ± 0.02	1.23 ± 0.03	nd	nd	nd	nd	nd	nd	Ouzouni et al., 2009
Armillaria mellea (Vahl) P. Kumm	0.61 ± 0.03	4.20 ± 0.05	nd	nd	nd	nd	nd	nd	Ouzouni et al., 2009
Armillaria tabescens (Scop.) Emel	0.14 ± 0.03	4.37 ± 0.06	nd	nd	nd	nd	nd	nd	Ouzouni et al., 2009
Boletus aureus Schaeff.	0.18 ± 0.02	0.86 ± 0.02	nd	nd	nd	nd	nd	nd	Ouzouni et al., 2009
Boletus griseus Forst	1.7 ± 1.1	2.8 ± 1.5	nd	nd	nd	670 ± 250	4600 ± 2400	440 ± 170	Liu et al., 2012b

(Continued)

Table 21.4 (Continued)

Mushroom Species	Magnesium (Mg)	Copper (Cu)	Manganese (Mn)	Iron (Fe)	Zinc (Zn)	Lead (Pb)	Cadmium (Cd)	Nickel (Ni)	References
Boletus speciosus Forst	1.0 ± 0.6	0.45 ± 0.31	nd	nd	nd	160 ± 53	2500 ± 1100	38 ± 20	Liu et al., 2012b
Cantharellus cibarius Fr.	0.05 ± 0.01	1.57 ± 0.04	nd	nd	nd	222	nd	nd	Ouzouni et al., 2009
Catathelasma ventricosum (Pk.) Singer	7.22 ± 0.05	5.34 ± 0.04	nd	nd	4820 ± 462	349 ± 21	27230 ± 2790	1973 ± 140	Liu et al., 2012a
Clitocybe maxima (Gartn. ex Mey.: Fr.) Quél	nd	nd	nd	0.05 ± 0.01	5390 ± 1930	1692 ± 376	26430 ± 4250	962 ± 293	Liu et al., 2012a
Clitocybe subconnexa (Murrill)	nd	nd	nd	nd	nd	7220.4 ± 233.7	20233 ± 1567.5	926.8 ± 9.6	Heleno et al., 2015b
Craterellus cornucopioides (L.: Fr.) Pers.	nd	nd	nd	nd	7130 ± 862	1185 ± 147	36620 ± 10030	1255 ± 241	Liu et al., 2012a
Fistulina hepatica Schaeff.: Fr.	0.18 ± 0.02	4.79 ± 0.02	nd	nd	nd	nd	nd	nd	Ouzouni et al., 2009
Hericium coraloides (Scop.) Pers.	nd	nd	nd	nd	nd	6780.4 ± 113.2	17788.7 ± 1393.7	837.5 ± 32.4	Heleno et al., 2015a
Hericium erinaceum (Bull.) Persoon	nd	nd	nd	nd	nd	5867.8 ± 22.0	11881 ± 131.4	443.5 ± 35.8	Heleno et al., 2015a
Hygrophorus russula (Schaeff.: Fr.) Quélet.	1.06 ± 0.02	1.38 ± 0.06	nd	nd	nd	nd	nd	nd	Ouzouni et al., 2009
Laccaria amethystea (Bull. ex Gray) Murr.	nd	nd	nd	0.72 ± 0.24	5040 ± 683	361 ± 96	25290 ± 6290	2004 ± 311	Liu et al., 2012a
Lactarius hygrophoroides Berk. et M.A. Curtis	2.1 ± 0.8	1.5 ± 0.9	nd	nd	nd	190 ± 66	3600 ± 1800	46 ± 34	Liu et al., 2012b
Lentinula edodes (Berk.) Pegler (cultivated)	nd	nd	1.82 ± 0.1	nd	7699 ± 641	3274 ± 516	13020 ± 1010	1749 ± 343	Mallikarjuna et al., 2012
Lentinus cladopus Leveille	nd	nd	1.9 ± 0.1	nd	1005 ± 23	nd	593 ± 102	1299 ± 261	Mallikarjuna et al., 2012
Lepista nuda (Bull.) Cooke	0.39 ± 0.03	0.59 ± 0.02	nd	nd	nd	nd	nd	nd	Ouzouni et al., 2009
Leucopaxillus giganteus (Sowerby) Singer	0.72 ± 0.17	6.3 ± 2.3	nd	nd	nd	350 ± 93	1400 ± 740	240 ± 75	Liu et al., 2012b
Macrocybe gigantea (Massee) Pegler et Lodge	0.29 ± 0.26	0.65 ± 0.37	nd	nd	nd	580 ± 94	1300 ± 450	470 ± 130	Liu et al., 2012b

Melanoleuca arcuata (Bull.) Singer	260 ± 120	2.5 ± 1.2	nd	nd	nd	450 ± 190	1300 ± 490	260 ± 120	Liu et al., 2012b
Morchella deliciosa Fr.	0.51 ± 0.22	5.9 ± 3.6	nd	nd	nd	440 ± 160	2100 ± 580	240 ± 84	Liu et al., 2012b
Mycena haematopus (Pers.) P. Kumm.	0.63 ± 0.28	1.5 ± 1.0	nd	nd	nd	190 ± 74	3200 ± 1100	120 ± 49	Liu et al., 2012b
Pleurotus djamor Sacc	nd	nd	1.1 ± 0.4	nd	7432 ± 298	616 ± 82	36340 ± 1220	342 ± 299	Mallikarjuna et al., 2012
Pleurotus florida (Block & Tsao)	nd	nd	4.8 ± 0.2	nd	6402 ± 80	305 ± 80	24720 ± 2070	82.7 ± 2	Mallikarjuna et al., 2012
Pulveroboletus ravenelii (Berk. et M.A. Curtis) Murrill	0.65 ± 0.30	5.6 ± 1.6	nd	nd	nd	250 ± 100	2700 ± 730	260 ± 56	Liu et al., 2012b
Ramaria largentii Marr & D.E. Stuntz	nd	nd	nd	nd	nd	nd	nd	nd	Ouzouni et al., 2009
Stropharia rugoso-annulata Farlow apud Murr.	0.05 ± 0.02	0.12 ± 0.04	nd	nd	7290 ± 1180	411 ± 65	16320 ± 9420	1371 ± 73	Liu et al., 2012a
Tricholoma matsutake (S. Itô et S. Imai) Singer	2.3 ± 1.8	2.6 ± 0.9	nd	nd	nd	270 ± 130	2400 ± 1300	270 ± 110	Liu et al., 2012b
	nd	nd	nd	nd	5040.0 ± 1.1	310.0 ± 0.6	23520 ± 4.4	410.0 ± 0.4	Liu et al., 2010
Volvopluteus gloiocephalus (DC.) Vizzini, Contu & Justo)	nd	nd	nd	nd	nd	7905.6 ± 672.6	46926.0 ± 644.3	735.2 ± 55.4	Heleno et al., 2015b

nd, not detected; dw, dry weight.

not found or were very low in the cultivated species *L. edodes* (Mallikarjuna et al., 2012). The results indicate that the mineral element contents in mushrooms are affected by the mushroom species and the environmental factors (Liu et al., 2012b).

Free amino acids are the main constituents of functionally essential compounds found in mushrooms (Kıvrak et al., 2014), participating in their flavor (Kalač, 2009). The content of free amino acids in mushrooms is low, only about 1% of dry matter. Their nutritional contribution is thus limited (Kalač, 2009). A total of 18 free amino acids were observed on the investigated mushrooms. The essential amino acids, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, valine, phenylalanine, and the non-essential amino acids, aspartate, glutamate, serine, glycine, cysteine, arginine, alanine, proline, and tyrosine were detected and quantifed. The results are summarized in Table 21.5.

The major diversity of amino acids was observed in *Boletus edulis* Bull. Fr., *Cantharellus tubaeformis* (Bull.) Fr., *Clavulina rugosa* (Bull.) J. Schröt., *Hydnum repandum* L., Fr, *L. laccata*, *Lepista nuda* (Bull.) Cooke, *Leucopaxillus giganteus* (Sowerby) Singer, *Russula rosea* Pers., *Tricholoma saponaceum* (Fr.) P. Kumm. (Ayaz et al., 2011) (18 known amino acids), while less diversity was observed in *Inonotus obliquus* (persoon) Pilat (Kim et al., 2009) (2 known amino acids). In general, the number of amino acids in mushrooms normally ranges between 14 and 18. Essential amino acids are crucial for mankind as the body cannot synthesize them, so they need to be obtained from foods. Mushrooms are an ideal source of essential amino acids (Kıvrak et al., 2014).

21.3 Bioactive Properties

The subject of "functional foods" is a hot topic in the recent research in which mushrooms can also be included. The Institute of Medicine's Food and Nutrition Board defines "functional foods" as foods or dietary components that provide health benefits beyond basic nutrition (Hasler, 1998; Ferreira et al., 2010). The functional properties of mushrooms depend on their bioactive molecules (Ferreira et al., 2010).

In Asian countries, specific mushroom species such as *Ganoderma lucidum* (Leyss.: Fr.) Karst.) and Shiitake (*Lentinus edodes* (Berk.) Sing.), have been used in folk medicine for centuries, being considered edible and medicinal resources (Wasser, 2002). Furthermore, hundreds of other mushroom species have been extensively studied for their biological properties and potential in the prevention and treatment of several human diseases (Lindequist et al., 2005; Chang, 2008; Ferreira et al., 2010; Guillamón et al., 2010; Xu et al., 2011; Alves et al., 2012, 2013; Taofiq et al., 2015). In the next sections, several bioactivities displayed by mushrooms will be highlighted.

21.3.1 Antioxidant Properties

Nowadays, there is a huge concern about oxidative damage since the planet is being more and more exposed to oxidative species, including free radicals, derived from bad routine habits and pollution exposure. A free radical is defined as any atom or molecule possessing unpaired electrons in the outer orbit, generally unstable, and very reactive causing oxidative stress to the human organism, leading to serious diseases such as cancer and cardiovascular problems (Gutteridge and Halliwell, 2000; Carocho and Ferreira, 2013). Maintaining a balance between the production of free radicals and the antioxidant defenses is a mandatory condition to successful operation of the human body (Ferreira et al., 2009; Carocho and Ferreira, 2013).

Table 21.5 Free amino acids (g/100 g dw) found in wild and cultivated mushroom species.

Mushroom Species	Threonine (Thr^{EAA})	Valine (Val^{EAA})	Methionine (Met^{EAA})	Isoleucine (Ile^{EAA})	Leucine (Leu^{EAA})	Phenylalanine (Phe^{EAA})	Lysine (Lys^{EAA})	Tryptophan (Trp^{EAA})	Histidine (His^{EAA})	References
Agaricus arvensis Schaeff	1.02 ± 0.20	0.99 ± 0.20	0.45 ± 0.01	1.59 ± 0.33	1.82 ± 0.36	0.94 ± 0.17	0.14 ± 0.27	0.47 ± 0.11	2.45 ± 0.54	Ayaz et al., 2011
Agaricus bisporus (J.E. Lange) Imbach (brown, cultivated)	0.761 ± 0.001	0.121 ± 0.007	0.060 ± 0.005	0.030 ± 0.004	0.021 ± 0.007	0.032 ± 0.009	0.50 ± 0.01	nd	0.165 ± 0.003	Kim et al., 2009
Agaricus blazei Peck	0.72 ± 0.07	0.17 ± 0.03	0.075 ± 0.005	0.08 ± 0.04	0.036 ± 0.006	0.010 ± 0.001	0.47 ± 0.06	nd	0.075 ± 0.007	Kim et al., 2009
Agrocybe chaxingu Huang	0.53 ± 0.06	0.51 ± 0.08	0.08 ± 0.01	0.39 ± 0.07	0.61 ± 0.11	0.29 ± 0.02	0.29 ± 0.07	nd	0.30 ± 0.02	Lee et al., 2011
Boletus edulis Bull. Fr.	1.04 ± 0.19	0.98 ± 0.20	0.89 ± 0.01	1.18 ± 0.22	1.6 ± 0.3	0.83 ± 0.16	1.16 ± 0.22	0.32 ± 0.06	0.55 ± 0.11	Ayaz et al., 2011
Cantharellus cibarius Fr.	0.48 ± 0.03	0.44 ± 0.02	0.09 ± 0.01	0.81 ± 0.06	0.87 ± 0.05	0.43 ± 0.03	0.53 ± 0.03	0.34 ± 0.03	0.32 ± 0.02	Ayaz et al., 2011
Cantharellus tubaeformis (Bull.) Fr	0.49 ± 0.02	0.41 ± 0.05	0.12 ± 0.01	0.94 ± 0.02	0.89 ± 0.02	0.44 ± 0.03	0.56 ± 0.03	0.117 ± 0.005	0.40 ± 0.02	Ayaz et al., 2011
Catathelasma ventricosum (Pk.) Singer	6.07 ± 0.06	18.52 ± 0.53	10.82 ± 0.94	0.22 ± 0.02	1.39 ± 0.26	2.44 ± 0.15	13.42 ± 0.49	12.19 ± 0.19	11.49 ± 0.52	Liu et al., 2012a
Clavulina rugosa (Bull.) J. Schröt.	0.70 ± 0.19	0.58 ± 0.97	0.13 ± 0.01	0.94 ± 0.01	0.97 ± 0.08	0.58 ± 0.08	0.73 ± 0.09	0.19 ± 0.02	0.37 ± 0.02	Ayaz et al., 2011
Clitocybe maxima (Gartn. ex Mey.: Fr.) Quél	7.24 ± 0.56	2.74 ± 0.46	nd	nd	0.44 ± 0.05	6.91 ± 0.34	0.79 ± 0.02	8.37 ± 0.67	27.34 ± 1.19	Liu et al., 2012a
Craterellus cornucopioides (L.: Fr.) Pers.	6.37 ± 0.58	0.41 ± 0.16	12.74 ± 1.03	0.08 ± 0.01	17.51 ± 0.48	nd	8.09 ± 0.61	nd	22.07 ± 3.39	Liu et al., 2012a
Flammulina velutipes (Curtis) Singer (cultivated)	0.64 ± 0.01	0.18 ± 0.02	0.01 ± 0.001	0.04 ± 0.03	0.05 ± 0.03	0.019 ± 0.006	0.62 ± 0.01	nd	0.24 ± 0.02	Kim et al., 2009
	0.22 ± 0.06	0.35 ± 0.04	0.06 ± 0.02	0.24 ± 0.04	0.38 ± 0.07	0.27 ± 0.03	0.15 ± 0.06	nd	0.13 ± 0.02	Lee et al., 2011
Ganoderma lucidum (Curtis) P. Karst.	0.01 ± 0.001	0.02 ± 0.003	0.01 ± 0.004	nd	nd	0.002 ± 0.003	0.01 ± 0.01	nd	nd	Kim et al., 2009
Hydnum repandum L., Fr.	0.55 ± 0.03	0.46 ± 0.02	0.11 ± 0.01	0.83 ± 0.09	0.95 ± 0.09	0.46 ± 0.01	0.51 ± 0.04	0.17 ± 0.01	0.41 ± 0.01	Ayaz et al., 2011

(Continued)

Table 21.5 (Continued)

Mushroom Species	Threonine (Thr^{EAA})	Valine (Val^{EAA})	Methionine (Met^{EAA})	Isoleucine (Ile^{EAA})	Leucine (Leu^{EAA})	Phenylalanine (Phe^{EAA})	Lysine (Lys^{EAA})	Tryptophan (Trp^{EAA})	Histidine (His^{EAA})	References
Inonotus obliquus (persoon) Pilat	nd	0.02 ± 0.01	nd	nd	nd	nd	nd	nd	nd	Kim et al., 2009
Laccaria amethystea (Bull. ex Gray) Murr.	12.82 ± 0.53	7.99 ± 0.61	2.59 ± 0.11	nd	16.83 ± 0.27	7.31 ± 0.83	11.97 ± 1.22	0.73 ± 0.05	0.72 ± 0.05	Liu et al., 2012a
Laccaria laccata (Scop.) Cooke	1.01 ± 0.08	0.80 ± 0.01	0.23 ± 0.01	0.637 ± 0.002	1.15 ± 0.06	0.74 ± 0.05	0.90 ± 0.06	0.36 ± 0.05	0.42 ± 0.02	Ayaz et al., 2011
Lentinus edodes (Berk.) Pegler (cultivated)	0.231 ± 0.005	0.041 ± 0.004	0.022 ± 0.004	0.027 ± 0.002	nd	0.005 ± 0.004	0.135 ± 0.008	nd	0.023 ± 0.002	Kim et al., 2009
Lepista nuda (Bull.) Cooke	1.01 ± 0.11	0.80 ± 0.12	0.28 ± 0.01	1.06 ± 0.08	1.38 ± 0.11	0.81 ± 0.09	1.07 ± 0.13	0.28 ± 0.04	0.68 ± 0.08	Ayaz et al., 2011
Leucopaxillus giganteus (Sowerby) Singer	1.32 ± 0.92	1.04 ± 1.04	0.41 ± 0.01	1.43 ± 0.08	1.72 ± 0.11	1.01 ± 0.07	1.33 ± 0.11	0.35 ± 0.03	0.93 ± 0.08	Ayaz et al., 2011
Pleurotus eryngii (DC.) Quél. (cultivated)	0.36 ± 0.02	0.08 ± 0.005	0.02 ± 0.03	0.02 ± 0.02	0.02 ± 0.01	0.009 ± 0.003	0.289 ± 0.009	nd	0.072 ± 0.002	Kim et al., 2009
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm. (cultivated)	0.71 ± 0.03	0.12 ± 0.01	0.004 ± 0.003	nd	0.032 ± 0.004	0.01 ± 0.01	0.36 ± 0.04	nd	0.46 ± 0.06	Kim et al., 2009
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm.	0.42 ± 0.02	0.44 ± 0.01	0.09 ± 0.01	0.33 ± 0.02	0.53 ± 0.03	0.29 ± 0.02	0.28 ± 0.00	nd	0.18 ± 0.01	Lee et al., 2011
Russula rosea Pers.	0.698 ± 0.009	0.647 ± 0.005	0.76 ± 0.01	0.93 ± 0.05	1.13 ± 0.03	0.595 ± 0.001	0.676 ± 0.009	0.112 ± 0.003	0.393 ± 0.007	Ayaz et al., 2011
Sparassis crispa	0.18 ± 0.02	0.05 ± 0.01	0.02 ± 0.01	0.001 ± 0.002	0.010 ± 0.007	nd	0.16 ± 0.04	nd	0.035 ± 0.001	Kim et al., 2009
Stropharia rugoso-annulata Farlow apud Murr.	7.44 ± 0.67	0.35 ± 0.04	31.94 ± 0.81	nd	nd	nd	7.11 ± 0.28	2.17 ± 0.21	12.13 ± 0.59	Liu et al., 2012a
Tricholoma saponaceum (Fr.) P.Kumm.	0.56 ± 0.05	0.55 ± 0.06	0.21 ± 0.01	0.76 ± 0.09	0.95 ± 0.12	0.51 ± 0.06	0.70 ± 0.09	0.20 ± 0.02	0.44 ± 0.05	Ayaz et al., 2011

Species	Aspartate (Asp^{NEAA})	Glutamate (Glu^{NEAA})	Serine (Ser^{NEAA})	Glycine (Gly^{NEAA})	Cysteine (Cys^{NEAA})	Arginine (Arg^{NEAA})	Alanine (Ala^{NEAA})	Proline (Pro^{NEAA})	Tyrosine (Tyr^{NEAA})	References
Agaricus arvensis Schaeff	1.75 ± 0.32	2.94 ± 0.53	0.99 ± 0.19	0.92 ± 0.22	0.32 ± 0.01	2.05 ± 0.47	1.67 ± 0.32	0.87 ± 0.15	0.34 ± 0.03	Ayaz et al., 2011
Agaricus bisporus (J.E. Lange) Imbach (brown, cultivated)	1.613 ± 0.003	1.80 ± 0.03	1.111 ± 0.005	0.59 ± 0.02	0.106 ± 0.006	0.038 ± 0.004	2.65 ± 0.02	0.85 ± 0.02	0.030 ± 0.003	Kim et al., 2009
Agaricus blazei Peck	0.74 ± 0.05	2.13 ± 0.22	0.79 ± 0.05	0.55 ± 0.03	0.17 ± 0.02	0.62 ± 0.09	2.89 ± 0.26	0.86 ± 0.05	0.025 ± 0.006	Kim et al., 2009
Agrocybe chaxingu Huang	0.56 ± 0.02	1.20 ± 0.12	0.58 ± 0.08	0.47 ± 0.05	nd	0.50 ± 0.05	1.03 ± 0.06	0.48 ± 0.05	0.19 ± 0.01	Lee et al., 2011
Boletus edulis Bull. Fr.	1.96 ± 0.39	2.79 ± 0.53	0.98 ± 0.18	0.86 ± 0.17	0.31 ± 0.01	1.13 ± 0.21	1.51 ± 0.29	0.85 ± 0.16	0.41 ± 0.07	Ayaz et al., 2011
Cantharellus cibarius Fr.	0.89 ± 0.06	1.50 ± 0.05	0.47 ± 0.04	0.39 ± 0.02	0.08 ± 0.01	0.79 ± 0.06	0.54 ± 0.03	0.43 ± 0.03	0.21 ± 0.01	Ayaz et al., 2011
Cantharellus tubaeformis (Bull.) Fr	0.96 ± 0.08	1.09 ± 0.18	0.46 ± 0.03	0.37 ± 0.02	0.13 ± 0.01	0.70 ± 0.03	0.49 ± 0.03	0.43 ± 0.02	0.38 ± 0.04	Ayaz et al., 2011
Catathelasma ventricosum (Pk.) Singer	nd	11.08 ± 1.07	7.67 ± 0.32	0.72 ± 0.14	2.37 ± 0.30	0.43 ± 0.13	1.17 ± 0.21	nd	nd	Liu et al., 2012a
Clavulina rugosa (Bull.) J. Schröt.	1.38 ± 0.16	2.79 ± 0.21	0.66 ± 0.07	0.55 ± 0.07	0.25 ± 0.01	1.45 ± 0.15	0.78 ± 0.09	0.57 ± 0.08	0.43 ± 0.08	Ayaz et al., 2011
Clitocybe maxima (Gartn. ex Mey.: Fr.) Quél	2.72 ± 0.83	0.90 ± 0.05	nd	11.69 ± 0.77	2.74 ± 0.18	0.08 ± 0.01	18.62 ± 2.74	nd	9.42 ± 0.48	Liu et al., 2012a
Craterellus cornucopioides (L.: Fr.) Pers.	0.54 ± 0.09	7.67 ± 0.49	16.79 ± 0.68	5.71 ± 0.13	0.34 ± 0.08	nd	0.86 ± 0.06	nd	0.82 ± 0.04	Liu et al., 2012a
Flammulina velutipes (Curtis) Singer (cultivated)	0.28 ± 0.01	3.15 ± 0.07	0.68 ± 0.04	0.61 ± 0.01	0.63 ± 0.03	0.13 ± 0.02	2.69 ± 0.05	0.90 ± 0.01	0.01 ± 0.02	Kim et al., 2009
	0.38 ± 0.07	1.00 ± 0.01	0.35 ± 0.05	0.34 ± 0.04	nd	0.23 ± 0.04	0.94 ± 0.1	0.39 ± 0.01	0.20 ± 0.02	Lee et al., 2011
Ganoderma lucidum (Curtis) P. Karst.	0.013 ± 0.002	0.019 ± 0.002	0.014 ± 0.001	0.06 ± 0.01	nd	nd	0.03 ± 0.01	nd	nd	Kim et al., 2009
Hydnum repandum L., Fr.	1.16 ± 0.05	1.34 ± 0.08	0.57 ± 0.03	0.40 ± 0.12	0.18 ± 0.01	0.63 ± 0.05	0.59 ± 0.03	0.46 ± 0.02	0.28 ± 0.06	Ayaz et al., 2011
Inonotus obliquus (persoon) Pilat	nd	nd	nd	nd	0.014 ± 0.001	nd	nd	nd	nd	Kim et al., 2009

(Continued)

Table 21.5 (Continued)

Mushroom Species	Threonine (Thr^{EAA})	Valine (Val^{EAA})	Methionine (Met^{EAA})	Isoleucine (Ile^{EAA})	Leucine (Leu^{EAA})	Phenylalanine (Phe^{EAA})	Lysine (Lys^{EAA})	Tryptophan (Trp^{EAA})	Histidine (His^{EAA})	References
Laccaria amethystea (Bull. ex Gray) Murr.	0.49 ± 0.03	13.12 ± 1.17	10.34 ± 0.26	2.20 ± 0.42	2.57 ± 0.94	nd	10.32 ± 1.92	nd	nd	Liu et al., 2012a
Laccaria laccata (Scop.) Cooke	1.49 ± 0.10	1.74 ± 0.12	0.87 ± 0.04	0.85 ± 0.07	0.27 ± 0.01	9.0 ± 0.13	0.83 ± 0.05	0.90 ± 0.11	0.555 ± 0.002	Ayaz et al., 2011
Lentinus edodes (Berk.) Pegler (cultivated)	0.200 ± 0.005	1.57 ± 0.02	0.16 ±	0.117 ± 0.001	0.842 ± 0.005	0.047 ± 0.006	0.472 ± 0.003	0.186 ± 0.003	0.012 ± 0.003	Kim et al., 2009
Lepista nuda (Bull.) Cooke	1.76 ± 0.18	2.85 ± 0.29	0.94 ± 0.09	0.78 ± 0.10	0.34 ± 0.01	0.91 ± 0.12	1.29 ± 0.15	0.73 ± 0.09	0.31 ± 0.06	Ayaz et al., 2011
Leucopaxillus giganteus (Sowerby) Singer	2.11 ± 0.03	3.76 ± 0.23	1.29 ± 0.19	1.08 ± 0.09	0.36 ± 0.01	1.51 ± 0.14	1.61 ± 0.14	0.89 ± 0.07	0.48 ± 0.06	Ayaz et al., 2011
Pleurotus eryngii (DC.) Quél. (cultivated)	0.47 ± 0.03	0.39 ± 0.02	0.41 ± 0.02	0.35 ± 0.02	0.05 ± 0.02	0.25 ± 0.01	0.86 ± 0.05	0.53 ± 0.02	0.03 ± 0.02	Kim et al., 2009
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm. (cultivated)	0.65 ± 0.02	3.69 ± 0.14	0.90 ± 0.03	0.325 ± 0.009	0.158 ± 0.003	0.24 ± 0.03	2.27 ± 0.09	0.47 ± 0.01	0.039 ± 0.003	Kim et al., 2009
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm.	0.56 ± 0.02	1.04 ± 0.01	0.54 ± 0.08	0.44 ± 0.02	nd	0.35 ± 0.02	0.80 ± 0.02	0.37 ± 0.02	0.20 ± 0.02	Lee et al., 2011
Russula rosea Pers.	1.18 ± 0.02	1.72 ± 0.19	0.67 ± 0.04	0.51 ± 0.01	0.18 ± 0.01	0.76 ± 0.02	0.83 ± 0.02	0.59 ± 0.02	0.289 ± 0.004	Ayaz et al., 2011
Sparassis crispa	0.42 ± 0.04	1.27 ± 0.16	0.47 ± 0.04	0.14 ± 0.01	10.005 ± 0.007	0.11 ± 0.06	0.54 ± 0.06	0.21 ± 0.01	nd	Kim et al., 2009
Stropharia rugoso-annulata Farlow apud Murr.	nd	22.96 ± 3.21	6.69 ± 0.25	0.63 ± 0.04	7.61 ± 0.11	nd	nd	nd	0.97 ± 0.21	Liu et al., 2012a
Tricholoma saponaceum (Fr.) P.Kumm.	1.12 ± 0.12	1.66 ± 0.18	0.56 ± 0.04	0.48 ± 0.06	0.25 ± 0.01	0.68 ± 0.07	0.74 ± 0.09	0.51 ± 0.06	0.23 ± 0.02	Ayaz et al., 2011

nd, not detected; dw, dry weight; EAA, essential amino acid; NEAA, non-essential amino acid; LOD, limit of detection.

Besides the endogenous antioxidant defenses such as antioxidant enzymes, the human body can be aided by external antioxidants that can be obtained by the inclusion of antioxidant foods in our diet, taking advantage of the health benefits of those molecules (Ferreira et al., 2009).

In this perspective, mushrooms have been extensively studied for their antioxidant properties for decades and are reported as strong antioxidant foods with strong potential in the combat of free radicals. This bioactivity is related to the bioactive compounds present in these organisms, especially phenolic acids, tocopherols, ascorbic acid, carotenoids, and polysaccharides, among others (Table 21.6) (Ferreira et al., 2009, 2014; Stajić et al., 2013).

The antioxidant activity of several mushroom species, the most abundant molecules responsible for this activity and the applied assays for antioxidant activity evaluation are presented in Table 21.6. The antioxidant properties have been evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, superoxide radical scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, ferrous ion chelating capacity, Folin Ciocalteu, β-carotene bleaching inhibition, Ferricianide Prussian blue and inhibition of lipid

Table 21.6 Antioxidant activity of mushroom species.

Mushroom Species	Antioxidant Activity Assay	Bioactive Compounds	References
Cordyceps sinensis (Berk.) Sacc.	DPPH scavenging activity, superoxide radical scavenging, ABTS radical scavenging, inhibition of lipid peroxidation	Polysaccharides	Yamaguchi et al., 2000
Antrodia camphorate (Zang & Su)	DPPH scavenging activity, ferrous ion chelating, inhibition of lipid peroxidation	Phenolic compounds, polysaccharides, tocopherols	Mau et al., 2004
Lepista nuda (Bull.: Fr.) Cooke	DPPH scavenging activity, inhibition of lipid peroxidation, reducing power	Phenolic acids, cinnamic acid, tocopherols	Elmastas et al., 2007
Agaricus brasiliensis Wasser, M. Didukh, *Boletus edulis* Bull., *Agrocybe cylindracea* (V. Brig.) Singer	DPPH scavenging activity, ferrous ion chelating, inhibition of lipid peroxidation	Phenolic compounds, polysaccharides, organic acids	Tsai et al., 2007
Hygrocybe conica (Scop.: Fr.) Kumm. *Pleurotus florida* Eger. *Pleurotus porrigens* (Pers.) Gillet *Polyporus tenuiculus* (Beauv.) Fr. *Schizophyllum commune* Fr.: Fr. *Xerula furfuracea* (Peck) Redhead	DPPH scavenging activity, reducing power, ferrous ion chelating, Folin Ciocalteu	Phenolic compounds, polysaccharides	Wong and Chye, 2009
Clitocybe. Alexandri (Gillet) Gillet *Lepista inversa* (Scop.: Fr.) Pat.	DPPH scavenging activity, Folin Ciocalteu, β-carotene bleaching inhibition, Ferricianide Prussian blue	–	Vaz et al., 2010

(Continued)

Table 21.6 (Continued)

Mushroom Species	Antioxidant Activity Assay	Bioactive Compounds	References
Clitocybe alexandri (Gillet) Konrad *Fistulina hepatica* (Schaeff.: Fr.) *Hydnum repandum* (L.: Fr.) *Hygrophoropsis aurantiaca* (Wulf.: Fr.) Mre. *Hypholoma capnoides* (Fr.) Quel. *Laccaria amethystina* (Bolt. Ex Fr.) R.Maire *Laccaria laccata* (scop.: Fr.) Berk. & Broome *Lactarius aurantiacus* (Fr.) *Lactarius salmonicolor* (Heim y Leclair) *Lepista inversa* (Scop.:Fr.) Pat. *Lepista sordida* (Fr.) Singer *Russula delica* (Fr.) *Russula vesca* (Fr.) *Suillus collinitus* (Fr.) Kuntz *Suillus mediterraneensis* (Jacquetant & Blum) Redeuilh	DPPH scavenging activity, Folin Ciocalteu, β-carotene bleaching inhibition	Tocopherols	Heleno et al., 2010
Boletus aereus Bull. *Boletus edulis* Bull.: Fr. *Boletus reticulatus* Schaeff.	DPPH scavenging activity, Folin Ciocalteu, β-carotene bleaching inhibition, Ferricianide Prussian blue	Phenolic acids, cinnamic acid, tocopherols	Heleno et al., 2012a
Armillaria mellea (Vahl) P. Kumm. *Calocybe gambosa* (Fr.) Donk *Clitocybe odora* (Fr.) P. Kumm. *Coprinus comatus* (O.F. Müll.) Pers.	DPPH scavenging activity, β-carotene bleaching inhibition, Ferricianide Prussian blue	Phenolic acids, cinnamic acid, tocopherols	Vaz et al., 2011
Boletus erythropus (Pers.) *Boletus fragrans* (Vittadini) *Calvatia utriformis* (Bull.) Jaap. *Clitopilus prunulus* (Scop. ex Fr.) P. Kumm *Hygrophorus pustulatus* (Persoon : Fries) Fries *Lycoperdon echinatum* Pers. *Lyophyllum decastes* (Fries: Fries) Singer *Macrolepiota excoriata* (Schaeff.) M.M. Moser *Russula cyanoxantha* (Schaeff.) Fr. *Russula olivacea* (Schaeff.) Fr.	DPPH scavenging activity, Folin Ciocalteu, β-carotene bleaching inhibition, Ferricianide Prussian blue	Tocopherols	Granjeia et al., 2011

Table 21.6 (Continued)

Mushroom Species	Antioxidant Activity Assay	Bioactive Compounds	References
Amanita caesarea (Scop.) Pers. *Cortinarius anomalus* (Fr.) Fr. *Cortinarius violaceus* (L.: Fr.) Gray *Lactarius volemus* (Fr.) Fr. *Suillus luteus* (L.: Fries) Gray	DPPH scavenging activity, β-carotene bleaching inhibition, Ferricianide Prussian blue	Phenolic acids, cinnamic acid, tocopherols	Reis et al., 2011
Auricularia auricular-judae (Bull.) Quel. *Pleurotus eryngii* (DC.:Fr.) Quél	DPPH scavenging activity, hydroxyl radical scavenging, inhibition of lipid peroxidation, ferrous ion chelating	Phenolic acids, ascorbic acid, polysaccharides	Oke et al., 2011
Agaricus campestris (L.) *Agaricus comtulus* Fries *Agaricus lutosus* (Møller) Møller *Amanita umbrinolutea* (Secr. ex Gillet) *Boletus armeniacus* Quél. *Boletus impolitus* Fr. *Bovista aestivalis* (Bonord.) Demoulin *Bovista nigrescens* (Pers.) *Chlorophyllum rhacodes* (Vittadini) Vellinga *Clavariadelphus pistillaris* L.:Fr.) Donk *Clavariadelphus truncatus* (Quel.) Donk *Clitocybe costata* Kühner & omagn *Clitocybe gibba* (Pers.) Kumm *Cortinarius praestans* Cordier *Flammulina velutipes* (Curtis) Singer *Hygrophorus chrysodon* (Fr.) Fr. *Leucoagaricus leucothites* Vittad. Wasser *Lycoperdon umbrinum* Pers. *Ramaria aurea* (Schaeff.) Quél. *Suillus Variegatus* (Sw.) Kuntze	DPPH scavenging activity, Folin Ciocalteu, β-carotene bleaching inhibition, Ferricianide Prussian blue	Tocopherols	Pereira et al., 2012
Suillus collinitus (Fr.) Kuntz	DPPH scavenging activity, β-carotene bleaching inhibition, Ferricianide Prussian blue	Phenolic acids, cinnamic acid	Vaz et al., 2012a

(Continued)

Table 21.6 (Continued)

Mushroom Species	Antioxidant Activity Assay	Bioactive Compounds	References
Coprinopsis atramentaria (Bull.) Redhead,Vilgalys & Moncalvo *Xerocomus chrysenteron* (Bull.) Quél *Ganoderma lucidum* (Curtis) P. Karst.	DPPH scavenging activity, β-carotene bleaching inhibition, Folin Ciocalteu, Ferricianide Prussian blue, TBARS	Phenolic acids, cinnamic acid, tocopherols	Heleno et al., 2012b
Morchella esculenta (L.) Pers.	DPPH scavenging activity, β-carotene bleaching inhibition, Folin Ciocalteu, Ferricianide Prussian blue, TBARS	Phenolic acids, cinnamic acid, tocopherols, organic acids	Heleno et al., 2013a
Macrolepiota procera (Scop.) Singer	DPPH scavenging activity, β-carotene bleaching inhibition, Folin Ciocalteu, Ferricianide Prussian blue, TBARS	Tocopherols	Fernandes et al., 2013b
Boletus edulis Bull.: Fr. *Hydnum repandum* L.: Fr.	DPPH scavenging activity, β-carotene bleaching inhibition, Folin Ciocalteu, Ferricianide Prussian blue, TBARS	Tocopherols, organic acids	Fernandes et al., 2013a
Agaricus bisporus (Lange) Imbach	DPPH scavenging activity, superoxide radical scavenging, inhibition of lipid peroxidation, reducing power	Phenolic acids, tocopherols polysaccharides	Liu et al., 2013
Agaricus blazei, Wasser, M. Didukh *Lentinus edodes* (Berk.) Pegler	DPPH scavenging activity, hydroxyl radical scavenging, reducing power, inhibition of lipid peroxidation	Phenolic acids, polysaccharides	Carneiro et al., 2013
Phellinus linteus (Berkeley & Curtis) Teng	DPPH scavenging activity, β-carotene bleaching inhibition, Ferricianide Prussian blue, TBARS	Polysaccharides, glucans, triterpenoids, phenolic acids, tocopherols	Reis et al., 2014
Amanita crócea (Quél. in Bourd.) Singer ex Singer *Amanita mairei* (Foley) *Boletus porosporus* (Imler ex Bon & G. Moreno) *Boletus regius* (Krombh.) *Gyromitra esculenta* (Pers. ex Pers.) Fr. *Helvella lacunosa* (Afzel.) *Russula aurea* Pers. *Russula virescens*(Schaeff.) Fr. *Boletus. Edulis* Bull. *Russula delica* Fr. *Agaricus albertii* (Bon.) *Agaricus urinascens var. excellens* (F.H. øller)	DPPH scavenging activity, β-carotene bleaching inhibition, Folin Ciocalteu, Ferricianide Prussian blue, TBARS	Phenolic acids. Cinnamic acid, tocopherols, organic acids	Leal et al., 2013; Fernandes et al., 2014; Reis et al., 2014a; Fernandes et al., 2015; Heleno et al., 2015b

Table 21.6 (Continued)

Pleurotus eryngii (DC:Fr.) Quélet
Amanita caesarea (Scop.) Pers.
Amanita curtipes Gilbert
Hericium erinaceus (Bull.) Persoon
Hericium coralloide s (Scop.) Pers.

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; TBARS, thiobarbituric acid reactive substances.

peroxidation by the thiobarbituric acid reactive substances (TBARS) assays. The studied mushroom species revealed strong antioxidant properties depending on the bioactive compounds found in samples. Mau et al. (2004) evaluated the antioxidant activity of two *Antrodia camphorata* mycelia (white and red) and reported the highest antioxidant activity for white mycelium presenting the lowest EC_{50} values (concentration providing 50% of antioxidant activity) ranging from 1.56 to 3.11 mg/mL in comparison with red mycelium (1.21–19.8 mg/mL). The authors attributed the antioxidant capacity to phenolics, tocopherols, and polysaccharides. In another study, Heleno et al. (2010) characterized several mushroom species for their content in phenolics and tocopherols, and also related these compounds to the observed activity of the mushroom extracts. The authors reported a strong capacity of *Hygrophoropsis aurantiaca* (Wulfen) Maire (EC_{50} values lower than 1.35 mg/mL) due to the contribution of phenolics (7.90 mg/g) and tocopherols (0.02–1.94 μg/g). Oke et al. (2011) evaluated the antioxidant activity of *Auricularia auricular-judae* (Bull.) Quel. and *Pleurotus eryngii* (DC.) Quél. that presented EC_{50} values ranging from 0.309 to 3.08 mg/mL, and 0.42 to 5.23 mg/mL, respectively, and was related to the phenolic acids and ascorbic acid contents. Several mushroom species from Portugal were analyzed by Pereira et al. (2012), and were described as sources of phenolic acids and vitamins that were related to the antioxidant capacity of the studied species. According to the studies performed by our research group, *Ganoderma lucidum* (Curtis) P. Karst, *Calvatia utriformis* (Bull.) Jaap, *Lyophillum decastes* (Fries: Fries) Singer are among the strongest antioxidant mushrooms by presenting the lowest EC_{50} values (0.10–0.26 mg/mL; 0.68–8.40 mg/mL; 0.29–0.98 mg/mL, respectively), followed by species from the genus *Boletus* (Heleno et al., 2012a,c; Grangeia et al., 2011).

The bioactive molecules present in mushrooms can be extracted and isolated to be used as nutraceuticals or in functional foods, for the combat of chronic diseases associated with oxidative stress. Furthermore, these compounds can also be included in our daily diet, promoting health and taking advantage of the synergistic effects of the present bioactives.

21.3.2 Antimicrobial Properties

The search for new antimicrobial agents has increased in recent years due to resistance developed by bacteria and fungi to antibiotics. Thus, the development of new formulations that can be effective in the treatment of bacterial and fungal diseases is crucial. From this perpective, mushrooms can also be an important source of antimicrobial molecules (Alves et al., 2012; Gyawali et al., 2014). There are several studies in the literature reporting the antimicrobial activity of mushrooms against Gram positive and Gram negative bacteria, and also against pathogenic fungi (Table 21.7). Most of the available studies report the antimicrobial activity of

Table 21.7 Antimicrobial activity of mushroom species.

Mushroom Species	Target Bacteria	Target Fungi	References
Lentinus edodes (Berk.)	Gram +: *Actinomyces naeslundii, Actinomyces viscosu, Bacillus cereus, Bacillus megaterium, Bacillus pumilis, Bacillus subtilis, Enterococcus faecalis, Enterococcus faecium, Lactobacillus casei, Listeria innocua, Listeria monocytogenes, Staphylococcus sp., Staphylococcus aureus, MRSA, Staphylococcus epidermidis, Streptococcus gordonii, Streptococcus mitis, Streptococcus mutans, Streptococcus oralis, Streptococcus pyogenes, Streptococcus salivarius, Streptococcus sanguinis, Streptococcus sobrinus, Micrococcus luteus.* Gram –: *Cupriavidis, Escherichia coli, Fusobacterium nucleatum, Klebsiella aerogenes, Klebsiella pneumonia, Neisseria subflava, Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Proteus mirabilis, Pseudomonas aeruginosa, Pseudomonas maltophila, Salmonella poona, Serratia marcescens, Veillonella díspar, Veillonella parvula, Yersinia enterecolitica.*	*Aspergillus fumigatus, Aspergillus niger, Scedosporium apiospermum*	Hatvani, 2001; Signoretto et al., 2009; Hearst et al., 2009; Alves et al., 2012; Alves et al., 2013
Clitocybe alexandri (Gillet) Gillet	Gram +: *B. cereus, B. subtilis, M. luteus.* *Gram –: Enterobacter aerogenes, E. coli.*	–	Solak et al., 2006
Laetiporus sulphureus (Bull.) Murrill.	Gram +: *S. aureus, B. subtilis, B. cereus, M. flavus, M. luteus.* Gram –: *P. aeruginosa, E. coli, Morganella morganii, Y. enterecolitica, P. vulgaris.*	*Candida albicans*	Türkoglu et al., 2007
Lactarius sp., L. deliciosus (L. ex Fr.) S.F.Gray, *L. piperatus* (L.) Pers., *L. volemus* (Fr.) Fr.,	Gram +: *B. cereus, B. subtilis, S. aureus.* Gram –: *E. coli, K. pneumonia, P. aeruginosa.*	*Cryptococcus neoformans, C. albicans*	Barros et al., 2007; Ozen et al., 2011
Boletus edulis Bull. Fr.	Gram +: *B. cereus, S. aureus.* Gram –: *E. coli, P. aeruginosa.*	–	Barros et al., 2008a
Cantharellus cibarius Fr.	Gram +: *B. cereus, B. subtilis, S. aureus.* Gram –: *E. coli, P. aeruginosa.*	*C. albicans*	Barros et al., 2008a; Barros et al., 2008b
Armillaria mellea (Vahl) P. Kumm	Gram +: *B. cereus, B. subtilis, S. aureus, Sarcina lutea.* Gram –: *E. cloacae, E. faecalis, E. coli, P. vulgaris, S. typhimurium.*	*C. albicans*	Kalyoncu and Oskay, 2008; Alves et al., 2012; Alves et al., 2013
Agaricus bisporus L., *A.bitorquis* (Quélet) Sacc., *A. bohusii* Bon, *A. silvícola* (Vittad.) Peck	Gram +: *B. cereus, B. subtilis, S. aureus, S. epidermidis, M. flavus, M. luteus.* Gram –: *E. aerogenes, E. coli, K. pneumonia, M. morganii, P. vulgaris, P. aeruginosa, S. typhi, S. typhimurium.*	*C. albicans, Candida tropicalis, Penicillium verrucosum*	Öztürk et al., 2011; Alves et al., 2012; Alves et al., 2013

Table 21.7 (Continued)

Mushroom Species	Target Bacteria	Target Fungi	References
Cortinarius sp.	Gram +: *B. cereus, B. subtilis, S. aureus.* Gram –: *E. coli, P. aeruginosa.*	*Trichophyton mentagrophytes, Cladosporium resinae, C. albicans*	Ozen et al., 2011
Hydnum repandum L., Fr	Gram +: *B. cereus, S. aureus.* Gram –: *E. coli, P. aeruginosa.*	*C. albicans*	Ozen et al., 2011
Ganoderma lucidum (Curtis) P. Karst	Gram +: *B. cereus, B. subtilis, E. cloacae, L. monocytogenes, S. aureus.* Gram –: *E. coli, K. pneumonia, P. aeruginosa, S. typhimurium.*	*A. fumigatus, Aspergillus versicolor, Aspergillus ochraceus, A. niger, Trichoderma viride, Penicillium funiculosum, Penicillium ochrochloron, P. verrucosum*	Heleno et al., 2013
Morchella esculenta (L.) Pers.	Gram +: *L. monocytogenes, S. aureus.* Gram -: *S. typhimurium,, E. coli, E. cloacae.*	–	Heleno et al., 2013a
Phellinus linteus (Berkeley & Curtis) Teng	Gram +: *S. aureus, B. cereus, M. flavus, L. monocytogenes.* Gram -: *P. aeruginosa, S. typhimurium, E. coli, E. cloacae.*	*A. fumigatus, A. versicolor, A. ochraceus, A. niger, T. viride, P. funiculosum, P. ochrochloron, P. verrucosum*	Reis et al., 2014
Auricularia cornea Ehrenb. *Calvatia gigantea* (Batsch) Lloyd *Hericium coralloides* (Scop.) Pers. *Pleurotus australis* (Cooke & Massee) Sacc. *Ileodictyon cibarium* Tul. & C.Tul.	Gram +: *B. cereus, B. subtilis,* S. *aureus.* Gram -: *P. aeruginosa, E. coli, Y. enterecolitica.*	–	Ren et al., 2014
Volvopluteus gloiocephalus (DC.) Vizzini, Contu & Justo) *Clitocybe subconnexa* (Murrill)	Gram +: *S. aureus, B. cereus, M. flavus, L. monocytogenes.* Gram –: *P. aeruginosa, S. typhimurium, E. coli, E. cloacae.*	*A. fumigatus, A. versicolor, A. ochraceus, A. niger, T. viride, P. funiculosum, P. ochrochloron, P. verrucosum*	Heleno et al., 2015a

(Continued)

Table 21.7 (Continued)

Mushroom Species	Target Bacteria	Target Fungi	References
Inonotus hispidus (Bull.) P. Karst., *Tricholoma caligatum* (Viv.) Ricken *Tricholoma columbetta* (Fr.) P.Kumm. *Phaeolus schweinitzii* (Fr.) Pat. *Xerocomus chrysenteron* (Bull.) Quél *Hydnellum ferrugineum* (Fr.) P.Karst. *Pleurotus ostreatus* (Jacq. ex Fr.) P.Kumm.	Gram +: *B. cereus.* Gram –: *P. aeruginosa*	–	Smolskaite et al., 2015

crude extracts; in recent research studies there are also reports of the antimicrobial activity of isolated compounds such as phenolic acids, peptides, and proteins (Alves et al., 2013a; Heleno et al., 2014a,b).

Among the studied species, *L. edodes* (Berk) seems to be the most studied one, presenting high activity against several pathogenic bacteria and fungi (Alves et al., 2012, 2013).

Analyzing Table 21.7, it can be observed that *L. edodes,* species from the *Agaricus* genus*, Cortinarius* sp., *G. lucidum, Volvopluteus gloiocephalus* (DC.) Vizzini, Contu & Justo*, Clitocybe subconnexa* (Murrill)*, Laetiporus sulphureus* (Bull.) Murrill*,* and *Phellinus linteus* (Berkeley & Curtis) Teng showed a strong activity against a large spectrum of pathogenic bacteria and fungi (Hatvani, 2001; Öztürk et al., 2011; Alves et al., 2012, 2013). Gram positive bacteria showed higher sensibility to the action of the tested mushroom extracts than Gram negative bacteria (Table 21.7). Several mushrooms were able to inhibit important pathogenic bacteria such as MRSA (methicillin-resistant *Staphylococcus aureus*), inhibited by *L. edodes* (Hatvani, 2001; Alves et al., 2013, 2013a), *Pseudomonas aeruginosa*, inhibited by the majority of the tested mushrooms, and *Listeria monocytogenes*, inhibited by *L. edodes*, species from the genus Agaricus, and *G. lucidum* among other mushrooms, and *Proteus mirabilis*, inhibited by *L. edodes* (Table 21.7).

Heleno et al. (2013b) studied the antibacterial activity of *G. lucidum* and verified that this mushroom presented high potential against the tested bacteria, namely *Staphylococcus aureus* and *Bacillus cereus* presenting minimal inhibitory concentrations (MIC) ranging from 0.0125–0.75 mg/mL and minimal bactericidal concentrations (MBC) in the range of 0.035–1.5 mg/mL, revealing stronger activity than the standards used (ampicillin and streptomicin). In another study, the authors verified that the extract of *Morchella esculenta* (L.) Pers. was effective against *Staphylococcus aureus*, presenting higher activity than the standards used (Heleno et al., 2013a). The authors related the observed activity with the presence of bioactive compounds, namely phenolic acids that also exhibited a promising antibacterial activity when tested individually (Alves et al., 2013a; Heleno et al., 2014b). *Fistulina hepatica* Schaeff.: Fr., *Leucopaxillus giganteus* (Sowerby) Singer, *Russula delica* (Fr.), and *Sarcodon imbricatum* (L.) P. Karst. were

also studied and the authors reported the ability of these extracts to potentiate the antibiotic action in the combat of pathogenic fungi (Alves et al., 2013b). The same mushroom species were also reported as being able to inhibit the biofilm formation of multirresistant bacteria *in vitro* (Alves et al., 2014).

Regarding antifungal activity, mushrooms have also proved their high capacity of inhibiting and/or killing different pathogenic fungi (Table 21.7). Species belonging to the *Agaricus* genus showed antifungal activity against *Candida albicans* and *Candida tropicalis* (Öztürk et al., 2011). The same authors and Hatvani (2001) reported that *L. edodes* showed activity against *Aspergillus fumigatus*, *A. niger*, *Candida albicans*, *C. krusei*, *C. parapsilosis*, and *Scedosporium apiospermum*. *G. lucidum* was also effective against different fungi presenting higher activity than the standards used (bifonazole and ketoconazole), *Trichoderma viride* being the most susceptible fungus (Heleno et al., 2013b).

Among the *Lactarius species*, *L. camphoratus* (Bull.) Fr. revealed activity against *C. albicans* (Ozen et al., 2011) and *L. sulphurous*, revealing a strong activity, even higher than the standard used, nystatin (Türkoglu et al., 2007).

From the available reports in the literature, *Candida* species are the most studied fungi, followed by *Aspergillus* and *Penicillium* species. Some colored conidiophores of fungi, such as *Aspergillus* and *Penicillium*, contain pigments belonging to the group of melanin: a green colored chromoprotein (Eisenman and Casadevall, 2012). Melanin is an important virulence factor contributing to the virulence of pathogens in humans. There are also studies reporting the demelanizing activity of mushroom species against fungi. *M. esculenta*, *G. lucidum* and *Coprinopsis atramentaria* (Bull.) Redhead, Vilgalys, and Moncalvo showed demelanizing activity against *Penicillium ochrochloron*, *Aspergillus niger*, *and A. fumigatus*, respectively (Heleno et al. 2013ab, 2014b). Although mushrooms have strong antimicrobial potential, further research should be conducted on the mechanism of action against pathogenic bacteria and fungi.

21.3.3 Antitumor Properties

The continuous increase of cancerous diseases has been a problem around the world; the number of cancer cases has been drastically increasing in the last century and the search for new drugs has been a priority in the research world. Natural matrices, namely mushrooms, have been playing an important role in this subject and have contributed to the discovery of new compounds capable of treating several cancer types and helping the immune system in this matter (Poucheret et al., 2006; Ferreira et al., 2010).

Among the available research there are already hundreds of mushroom species described as capable of inhibiting the growth of human tumor cell lines, as well as bioactive compounds isolated from these organisms individually (Wasser and Weis, 1999; Clericuzio et al., 2004; Ferreira et al., 2010; Vaz et al., 2012b).

Mushrooms contain a huge diversity of bioactive molecules such as polysaccharides, lectins, sesquiterpenes, triterpenoids, proteins, glycoproteins among others (Table 21.8) that can be a source of powerful new pharmaceutical products in cancer treatment (Ye et al., 2005; Carbonero et al., 2012; Reis et al., 2014). Besides these bioactive compounds, crude extracts have been reported as also capable of preventing tumorigenesis, supressing cancer growth, especially in known cancer types such as stomach, lung, and breast cancers (Kanokmedhakul et al., 2012; Wang et al., 2012; Zhang et al., 2014; Radzki et al., 2016).

Table 21.8 presents the antitumor activity of different mushroom species and compounds isolated that revealed this bioactivity. Ye et al. (2005) isolated grifolin, a bioactive compound from *Albatrellus confluens* (Alb. & Schwein.) Kotl. and Pouzar and described its capacity to

Table 21.8 Antitumor activity of mushroom species.

Mushroom Species	Bioactive Extracts or Compounds	Bioactivity Displayed	References
Phellinus rimosus (Berk) Pilat.	Ethyl acetate, methanol, and aqueous extracts	Cytotoxic activity against DLA and EAC tumor cell lines	Ajith et al., 2003
Volvariella volvacea (Bulliard ex Fries) Singer	Lectin	Activation of cyclin-dependent kinase inhibitors, against S180 mouse sarcoma cells	Liu et al., 2001
Albatrellus confluens (Alb. & Schwein.) Kotl. & Pouzar	Grifolin	Inhibition of human cancer cell lines: CNE1, HeLa, MCF7, SW480, K562, Raji and B95–8marmoset B lymphoblastoid	Ye et al., 2005
Lentinula edodes (Berk.)	Ethanolic extract	Reduction of cell proliferation and induction of apoptosis against CH72 cells, cell cycle arrest by induction of G1 arrest	Gu et al., 2005
Pholiota spumosa (Fries) Singer	Putrescine-1,4-dicinnamide	Inhibition of cell growth PCA tumor cells by inducing apoptosis	Russo et al., 2007
Lentinula edodes (Berk.)	Methanolic and aqueous extracts	Cytostatic and cytotoxic activity against MCF-7 tumor cell line	Israilides et al., 2008
Suillus placidus (Bonord.) Singer	Suillin	Induction of apoptosis in HepG2 cells	Liu et al., 2009
Clitocybe nebularis (Batsch), P.Kumm.	Ricin B-like lectins	Inhibition of proliferation of leukemic T cells	Pohleven et al., 2009
Amanita virosa Secr.	Hemolytic lectin, toxovirin	Cytotoxic activity against tumor L1210 cells and human leukemia T cells	Antonyuk et al., 2010
Agaricus blazei Murrill	Agaritine	Antiproliferative activity on leukemic cell lines (U937, MOLT4, HL60, and K562)	Endo et al., 2010
Ganoderma lucidum (Curtis) P. Karst.	Ethanolic extract	Indution of apoptosis on human AGS tumor cells	Jang et al., 2010
Clitocybe alexandri (Gillet) Gillet	Ethanolic extract	Cytotoxic activity against MCF7, NCI-H460, HCT15 cancer cells Induction of apoptosis on lung cancer (NCI-H460) cells by S-phase cell cycle arrest	Vaz et al., 2010;Vaz et al., 2012b
Suillus collinitus (Fr.) Kuntz	Ethanolic extract	Cytotoxic activity against MCF7, NCI-H460, HCT15 cancer cells	Vaz et al., 2012a
Neonothopanus nambi Speg.	Sesquiterpenes	Cytotoxic activity against tumor NCI-H187 and KKU cell lines	Kanokmedhakul et al., 2012

Table 21.8 (Continued)

Mushroom Species	Bioactive Extracts or Compounds	Bioactivity Displayed	References
Pleurotus sajor-caju Fr.	β-glucan	Immunostimulatory effects by the activation of RAW 264.7 macrophages and induction of the synthesis of NO and cytokines TNF-α, IL-β on RAW 264.7 cell line	Carbonero et al., 2012
Flammulina velutipes (Curt.:Fr.) Sing.	Sesquiterpenoids	Cytotoxicity against HepG2, MCF-7, SGC7901, and A549 tumor cells	Wang et al., 2012
Sarcodon aspratus (Berk.)	Polysaccharide	Cytotoxic activity against HeLa	Chen et al., 2013
Pleurotus sajor-caju Fr.; *Lentinus edodes* (Berk.)	Aqueous extracts	Cytotoxic activity against Hep-2and HeLa tumor cells	Finimundy et al., 2013
Hypsizygus marmoreus (Peck) Bigelow	Glycoprotein	Inhibitory activity of human leukemic U937 cells	Tsai et al., 2013
Suillus luteus (L.) Roussel	Methanolic extract	Inhibition of growth and proliferation MCF-7, NCI-H460, AGS, and HCT-15 tumor cells	Santos et al., 2013
Phellinus linteus (Berkeley & Curtis) Teng	Methanolic and ethanolic extracts, polysaccharides, glucans, triterpenoids	Cytotoxic activity against tumor cell lines: MCF-7, NCI-H460, HCT-15, HeLa, and HepG2	Reis et al., 2014
Pholiota nameko (T. Itô) S. Itô & S. Imai	Protein (PNAP)	Cytotoxic activity against MCF-7 and HeLa tumor cells	Zhang et al., 2014
Coprinopsis atramentaria (Bull.: Fr.) Redhead, Vilgalys & Moncalvo	Methanolic extract	Cytotoxic activity against MCF7, NCI-H460, HCT15 cancer cells	Heleno et al., 2014
Fomes fomentarius	Polysaccharide	Inhibition of A549 cells growth	Kim et al., 2015
Inonotus obliquus (Fr.)	Aqueous and ethanolic extracts	Cytotoxic activity against MCF-7, NCI-H460, HeLa, and HepG2 tumor cell lines	Glamočlija et al., 2015
Inonotus Obliquus (Fr.)	Ergosterol peroxide	Antiproliferative and apoptotic activities on colon cancer cells (HCT116,HT-29,SW620 and DLD-1 CRC), suppression of clonogenic colony formation	Kang et al., 2015
Astraeus asiaticus Phosri, M.P.Martín & Watling	Lanostane triterpenoid Derivatives	Cytotoxic activity against human epidermoid carcinoma (KB) and human small cell lung cancer (NCI-H187)	Pimjuk et al., 2015

(Continued)

Table 21.8 (Continued)

Mushroom Species	Bioactive Extracts or Compounds	Bioactivity Displayed	References
Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm.	Polysaccharide	Antiproliferative activity against tumor MCF-7 and T-47D cells	Radzki et al., 2016

A549: human lung carcinoma; HepG2, human hepatoma cells; CNE1, human nasopharyngeal carcinoma cells; HeLa, human cervical carcinoma cells; MCF7, human breast carcinoma cells; SW480, human colon carcinoma cells; K562, human chronic myelogenous leukemia cells, Raji: human Burkitt's lymphoma cells; B95–8: marmoset B lymphoblastoid cells; NCI-H460, non-small cell lung cancer cells, HCT-15, colon carcinoma cells; T-47D, human breast carcinoma cells; NCI-H187, human small lung cancer cells; KKU, cholangiocarcinoma cell lines; L1210, murine leukemia cells; HeLa, uterine cervix carcinoma cells; RAW 264.7, mouse leukemic monocyte macrophage cells; Hep-2, laryngeal carcinoma tumor cells; CH72, murine skin carcinoma cells; SGC7901, human gastric carcinoma cells, A549, human lung carcinoma cells; DLA, Dalton's lymphoma ascites; EAC, Ehrlich's ascites carcinoma; PCA, human prostate cancer cells; AGS, gastric cancer cells.

induce apoptosis in several human cancer cell lines (nasopharyngeal carcinoma (CNE1), cervical carcinoma (HeLa), breast carcinoma (MCF7), colon carcinoma (SW480), chronic myelogenous leukemia (K562), Burkitts Lymphoma (Raji) and marmoset B lymphoblastoid (B95–8)). In another study, polysaccharides from *Fomes fomentarius* were also reported as inhibitors of human lung carcinoma cells (A549) (Kim et al., 2015). Also β-glucans, triterpenoids, proteins, algaritine, ergosterol peroxide, and glycoproteins were reported as strong antitumor agents isolated by *Pleurotus sajor-caju* Fr., *Phellinus linteus, Pholiota nameko* (T. Itô) S. Itô & S. Imai, and *Agaricus blazei* Peck., *I. obliquus*, and *Hypsizygus marmoreus* (Peck) Bigelow have also been reported as potent antitumoral agents by potentiating the immune system, inducing apoptosis, or inhibiting the growth of tumor cell lines (Table 21.8) (Endo et al., 2010; Carbonero et al., 2012; Tsai et al., 2013; Reis et al., 2014; Zhang et al., 2014; Kang et al., 2015). Besides isolated compounds, the crude extracts are also strong antitumor agents taking advantage of the synergistic effects of the present bioactive molecules. Methanolic, aqueous, and ethanolic extracts of *L. edodes*, *I. obliquus*, *P. linteus*, *Suillus luteus* (L.: Fries) Gray, *C. atramentaria*, *G. lucidumi, C. alexandri*, *Suillus collinitus* (Fr.) Kuntz, and *P. linteus* also revealed cytotoxicity against a wide range of tested tumor cell lines (Table 21.8) (Gu et al., 2005; Israilides et al., 2008; Jang et al., 2010; Vaz et al., 2010, 2012a, 2012b; Santos et al., 2013; Heleno et al., 2014a; Reis et al., 2014; Glamočlija et al., 2015). Furthermore, the analyzed compounds and extracts presented low or no toxicity against normal liver cells. According to the studies performed by our research group, the species *S. collinitus*, *S. luteus*, and *C. alexandri* revealed the highest activity against MCF7 cell line; *C. atramentaria* against NCI-H460 cell line; *S. luteus* against HCT15 cell line; and *S. collinitus* against HepG2 cell line. Moreover, the tested extracts showed no toxicity against normal cells of porcine liver at the maximum tested concentrations.

21.3.4 Other Bioactivities

Besides antioxidant, antimicrobial, and antitumor properties, it is also necessary to highlight other important bioactivities reported for mushroom extracts and compounds.

There are also a few studies on the anti-inflammatory activity of different mushroom species and bioactive compounds present in the extracts. Moro et al. (2012) studied *A. bisporus*, *Cantharellus cibarius* (Fr.), and *Lactarius deliciosus* (L. ex Fr.) S. F. Gray methanolic extracts and reported that these extracts were able to inhibit NO production in 30, 70, and 40%,

respectively, at 0.5 mg/mL in lipopolysaccharide (LPS) activated RAW 264.7 macrophages. In another study, *Cordyceps militaris* (L.) Fr. aqueous extract inhibited NO production, iNOS mRNA, and protein expression in colon tissue of dextran sodium sulfate (DSS)-induced colitis and in LPS-stimulated RAW264.7cells (Han et al., 2011). Taofiq et al. (2015) studied the anti-inflammatory activity of several mushroom species and reported that *Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm., *Macrolepiota procera* (Scop.) Singer, *Boletus impolitus* (Fr.), and *A. bisporus* ethanolic extracts showed the strongest anti-inflammatory potential with EC_{50} values ranging from 96 to 190 μg/mL. The authors related the observed activity to the high content of cinnamic acid found in the mentioned species. Besides the crude extracts, individual compounds were also studied for the same bioactivity, especially polysaccharides. A β-*D*-glucan isolated from *Pleurotus sajor-caju* exhibited immunomodulatory activity on human monocytic THP-1 macrophages, caused the inhibition of the inflammatory phase of nociception induced by formalin in mice, and finally was also able to reduce the number of total leukocytes and myeloperoxidase levels induced by LPS (Silveira et al., 2014). Different authors studied other compounds such as triterpenes and sterols isolated from *G. lucidum* and *L. polychrous*, respectively, and reported a strong inhibition of NO production (Dudhgaonkar et al., 2009; Fangkrathok et al., 2013).

Mushrooms have also been studied as food preservers, showing promising activities in this area. Stojković et al. (2014) reported the antioxidant and antimicrobial properties of *A. bisporus* and *A. brasiliensis* and the preserving capacity of these extracts in yogurts. *B. edulis* hydrophilic extracts were also incorporated in beef burgers and proved able to protect the product from oxidation during storage (Barros et al., 2011). Ribeiro et al. (2015) analyzed the synergistic effects of the species *S. luteus* and *C. atramentaria*, with the objective of incorporating the optimized proportions in cottage cheese. The authors verified that the encapsulated extracts were able to preserve the antioxidant activity of cottage cheese over the time.

21.4 Conclusions

Mushrooms are rich sources of nutrients, namely carbohydrates and proteins, presenting low fat content and for that reason these interesting organisms are appropriate for inclusion in low calorie diets. The chemical characterization of mushrooms and important bioactivities displayed highlight the importance of the inclusion of mushrooms in our daily diet, and of their potential as functional foods. Moreover, taking into account the promising biological activities exhibited by mushroom extracts and their isolated compounds, several species can be considered as functional food sources of nutraceuticals.

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22

Medicinal Properties and Clinical Effects of Medicinal Mushrooms

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22.1 Introduction

The use of mushrooms in traditional ancient therapies dates back at least to the Neolithic age. For millennia, mushrooms have been valued as edible and medical provisions for humankind. Contemporary research has validated and documented much of the ancient knowledge on medicinal mushrooms (MM). The interdisciplinary field of science that studies MMs has been developed and increasingly demonstrates potent and unique properties of compounds extracted from a range of mushroom species in the last three decades. Modern clinical practice in Japan, China, Korea, Russia, and several other countries relies on mushroom-derived preparations (Reshetnikov et al., 2001; Van Griensven, 2009; Wasser, 2010; Chang and Wasser, 2012)

A long history of mushrooms usage has been documented in Europe (e.g., the story of Ice Man, or Otzi's mushrooms: *Piptoporus betulins* – birch polypore and *Fomes fomentarius* – tinder conk, and *Amanita muscaria* – fly agarics in Celtic myths), in Asia (especially for lingzhi or reishi mushroom – *Ganoderma lucidum* in China; for *A. muscaria* – fly agaric in Russia, in Tibetian shamanism, and Buddhism, for *Lentinus edodes* – Shiitake mushroom in Japan); use of *Phellinus igniarius* by the Eskimos of Alaska, and some other species in on the African continent (for instance, mushrooms of Yoruba populations in Nigeria and Benin, in Algeria, and Egypt). Hallucinogenic species of the genus *Psilocybe* occupy a very special place in cultures of Mesoamerica, in Mexico and Guatemala (Wasson, 1968; Reshetnikov et al., 2001; Poder, 2005; Van Griensven, 2009; Chang and Wasser, 2012).

Nowadays, MMs are used as: (1) dietary food (world mushroom production was 33 million tons in 2014); (2) dietary supplement (DS) products (the market of MM DS products is rapidly growing and comprises a value of more than 18 billion US dollars per year); (3) a new class of drugs called "Mushroom Pharmaceuticals"; (4) natural bio-control agents in plant protection demonstrating insecticidal, fungicidal, bactericidal, herbicidal, nematocidal, and antiphytoviral activities; and (5) cosmeceuticals – different compounds of MMs including polysaccharides, such as soluble β-glucans, GXM, sacchachitin, tyrosinase, and other enzymes are used by cosmetic companies for their film-forming capability, activation of epidermal growth factor, antioxidative, antiallergic, antibacterial and anti-inflammatory activities, stimulation of collagen activity, inhibition of autoimmune vitiligo, and treating acne (Hyde et al., 2010; Wasser, 2010; Chang and Wasser, 2012).

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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MM are comparable to "medicinal plants" and can be defined as macroscopic fungi, mostly higher Basidiomycetes and some Ascomycetes, which are used in the form of extracts or powder for prevention, alleviation, or healing multiple diseases, and/or in balancing a healthy diet. According to the definition of "herbal drugs," dried fruit bodies, mycelia, or spores are considered "mushroom drugs" or "fungal drugs." Analogous to "phytopharmaceuticals" or "herbal preparations," the resulting mushroom preparations should be considered as "mushroom pharmaceuticals" or "mushroom preparations." The clear advantages of using mushroom-based products with regard to safety (as opposed to herbal preparations) are the following:

1) The overwhelming majority of mushrooms used for production are cultivated commercially, and not gathered in the wild. This guarantees proper identification and pure, unadulterated products. In many cases, it also means genetic uniformity.
2) Mushrooms are easily propagated vegetatively and thus keep to one clone. The mycelium can be stored for a long time, and the genetic and biochemical consistency may be checked after a considerable time.
3) The main advantage might be the fact that many mushrooms are capable of growing in the form of mycelial biomass in submerged cultures (Hawksworth, 2001; Wasser, 2010; Chang and Wasser, 2012; Lindequist, 2013).

Mushrooms and fungi in general are extremely abundant and diverse worldwide. Recent estimates of the number of fungi on Earth range from 500,000 to more than 5 million species, with a widely accepted working figure around 1.5 million, published more than 20 years ago. To date, it is recommended that as many as 3 million species of fungi should be accepted for general application (Hawksworth, 2012). Meanwhile, the total number of described fungi of all kinds is currently 110,000 species. The figure is based on the total reached by adding the number of species to each genus given in the last edition of the *Dictionary of Fungi* (Kirk et al., 2008) and other recent publications (Bass and Richards, 2011; Blackwell, 2011; Hawksworth, 2012), and includes all organisms traditionally studied by mycologists: slime molds, chromistan fungi, chytridiaceous fungi, lichen-forming fungi, filamentous fungi, molds, and yeasts. Out of these, mushrooms constitute 16,000 species, calculated from the *Dictionary of Fungi* (Kirk et al., 2008) and other publications of recent years. The number of mushroom species on Earth is currently estimated at 150,000–160,000, so only around 10% of existing mushroom species are known to science so far (Hawksworth, 2001, 2012; Kirk et al., 2008; Wasser, 2010). An analysis of the localities, from which fungi new to science have been described and cataloged in the Index of Fungi in the last 10 years, revealed that about 60% of all newly described fungi are from the tropics. This is also the case for mushrooms, especially those species forming ectomycorrhizas with native trees, although new species continue to be discovered in Europe and North America. In various tropical areas, 22–55% (in some cases up to 73%) of mushroom species have not yet been described (Hawksworth, 2001, 2012; Bass and Richards, 2011). Modern sequencing methods suggest that as many as 5 million species of fungi exist (Blackwell 2011). Therefore, we would need more than 4,000 years to describe this fungal diversity based on the present discovery rate of about 1,200 new species for science per year, which was the average for the last 10 years (Hibbett and Taylor, 2013). Summarizing these data we can assume that approximately 2% of world fungal biota and around 10% of world mushroom biodiversity were discovered by mycologists to date, thus the bulk of fungal biodiversity still remains hidden (Hawksworth, 2001; Wasser, 2010; Chang and Wasser, 2012).

Specialists in taxonomy of some groups of MM are familiar with "known" species, but some of their biochemical and pharmacological properties remain hidden. Wasser (2010) and Hawksworth (2012) have summarized the data on approximately 700 mushroom species with

pharmacological properties out of 2,000 known safe species. It can be seen that the current state of knowledge presents a great potential for MM diversity.

Mushrooms are currently evaluated for their nutritional value. They are rich in proteins, chitin (dietary fibers), vitamins, and minerals, low in total fat but with a high proportion of unsaturated fatty acids, and have no cholesterols. As for the characteristics of taste, mushrooms serve as a delicious foodstuff and also as a source of food flavoring substances (because of their unique flavors). In addition to the volatile eight-carbon compounds, the typical mushroom flavor consists of water-soluble taste components such as soluble sugars, polyols, organic acids, free amino acids, and 5-nucleotides.

Regarding the beneficial nutritional effects of mushrooms, the following facts should be noted:

1) Mushrooms are low in calories, which is beneficial for weight reduction.
2) Mushrooms have a in significant levels of purine, which are beneficial for the diet of people suffering from metabolic diseases.
3) Mushrooms have a low glucose level, and more mannitol, which makes them highly suitable for diabetics.
4) Mushrooms have a very low sodium concentration, which is beneficial for the diet of people suffering from high blood pressure.
5) Mushrooms have a high content of several key vitamins, which is an important orthomolecular aspect. This means that a significant part of the daily requirement in different essential vitamins can be covered by consuming mushrooms.
6) Mushrooms have a high content of potassium and phosphorus, which is an important orthomolecular aspect as well.
7) Finally, mushrooms have a high content of selenium, which is regarded as an excellent antioxidant.

Pharmacological properties of mushrooms are currently widely recognized. They make up a vast and yet largely untapped source of powerful new pharmaceutical products. In particular, and most importantly for modern medicine, MMs present an unlimited source of polysaccharides (especially β-glucans) and polysaccharide-protein complexes with anticancer and immunostimulating properties. Many, if not all, higher Basidiomycetes mushrooms contain different types of biologically active high-molecular-weight and low-molecular-weight compounds (triterpenes, lectins, steroids, phenols, polyphenols, lactones, statins, alkaloids, and antibiotics) in fruit bodies, cultured mycelia, and cultured broth (Anke, 1989; Hawksworth, 2001; Zaidman et al., 2005; Wasser, 2010; Chang and Wasser, 2012; De Silva et al., 2013).

22.2 Current Perspectives and Advances

There is a total of 200 medicinal functions produced by MMs and fungi. Recently studied medicinal actions of mushrooms included antitumor, immunomodulating, antioxidant, radical scavenging, cardiovascular, cholesterol-lowering, antiviral, antibacterial, anti-parasitic, antifungal, detoxicative, hepatoprotective, anti-diabetic, anti-obesity, neuroprotective, neuroregenerative, and some other effects. Also, substances derived from MMs can be used as painkillers and analgesics. The best implementation of MMs drugs and MM DSs has been in preventing immune disorders and maintaining good quality of life, especially in immunodeficient and immunodepressed patients, patients undergoing chemotherapy or radiotherapy, patients with different types of cancers, chronic blood-borne viral infections of hepatitis B, C, and D, different types of anemia, the human immunodeficiency virus/acquired immunodeficiency

syndrome (HIV/AIDS), *Herpes simplex* virus (HSV), chronic fatigue syndrome, Epstein–Barr virus, patients with chronic gastritis and gastric ulcers caused by *Helicobacter pylori*, and people suffering from dementia (especially Alzheimer's disease) (Dai et al., 2009; Wasser, 2010; Lo and Wasser, 2011; Chang and Wasser, 2012).

Mushroom polysaccharides prevent oncogenesis, show direct antitumor activity against various synergetic tumors, and prevent tumor metastasis. Their activity is especially beneficial when used in conjunction with chemotherapy. The antitumor action of polysaccharides requires an intact T-cell component; their activity is mediated through a thymus-dependent immune mechanism. They activate cytotoxic macrophages, monocytes, neutrophils, natural killer cells, dendritic cells, and chemical messengers (cytokines, such as interleukins, interferons, and colony stimulating factors) that trigger complementary and acute phase responses. Also, mushroom polysaccharides can be considered as multi-cytokine inducers able to induce gene expression of various immunomodulating cytokines and cytokine receptors (Zhang et al., 2007, 2011, 2013; Wasser, 2010; Lee and Kim, 2014; Rahman et al., 2015).

Cancer, most likely, has always existed in mankind; it is probably as old as life. Evidence of cancer was reported in a Neanderthal skull (35,000 BC) and Egyptian and Incan mummies (Barillot et al., 2013). Cancer is a broad term that includes hundreds of different types of diseases that can develop in the body. Cancer is a generic term used for malignant neoplasms.

The figures and projections of the global cancer burden presented in the new edition of the World Cancer Report (Stewart and Wild, 2014) starkly highlight the problem: the incidence of cancer has increased from 12.7 million in 2008 to 14.1 million in 2012, and this trend is projected to continue, with the number of new cases expected to increase in the future by 75%. Among men, the five most common sites of cancer diagnosed in 2012 were the lung (16.7% of the total), prostate (15.0%), colorectum (10%), stomach (8.5%), and liver (7.5%). Lung cancer had the highest incidence (34.2 per 100,000) and prostate cancer had the second highest incidence (31.1 per 100,000). Among women, the five most common sites of cancer were the breast (25.2% of the total), colorectum (9.2%), lung (8.7%), cervix (7.9%), and stomach (4.8%). Breast cancer had a substantially higher incidence (43.3 per 100,000) than any other cancer; the next highest incidence was colorectal cancer (14.3 per 100,000). Among the four major noncommunicable diseases – cardiovascular diseases, chronic diabetes, respiratory diseases, and cancer at the national, regional, and global levels, it is cancer that is now the main cause of death around the world in the last few years (Stewart and Wild, 2014).

A total of more than 40 million new cancer cases were predicted in the world for 2015. The estimates reported by WHO indicate that 84 million people should have died of cancer between 2005 and 2015. Cancer is killing more people than AIDS, malaria, and tuberculosis combined. Additionally, in China and India (the most populated countries), cancer deaths are increasing, largely because of smoking, unhealthy and unbalanced diet, and ecology problems. Globally, the number of deaths from cancer, according to WHO, should reach 17 mn/per year in 2030 (Stewart and Wild, 2014).

Medicinal mushroom drugs and MM polysaccharides preparations from different species show positive results in treating cancers *in vitro* and *in vivo*. A new class of antitumor MM drugs has been called biological response modifiers (BRMs). The application of BRMs has become the new kind of cancer treatment together with surgery, chemotherapy, and radiotherapy (Mizuno, 1999; Wasser, 2010; Zhang et al., 2011, 2013; Chang and Wasser, 2012; Lee and Kim, 2014). The major problems associated with cancer treatments, especially chemotherapy and radiotherapy, are damage to or weakening of the patient's natural immunological defenses. Medicinal mushroom BRMs help treat cancers – focusing on improving the patient's quality of life, since they significantly reduce the side effects and help overcome cancer

growth. Most of them activate natural immune responses of the host and can be used as supportive treatment for cancer prevention and, in some cases, alone with conventional therapies.

22.3 Medicinal Mushroom Drugs

Immunoceuticals isolated from more than 30 MM species have demonstrated antitumor activity in the studies done on animals. However, only a few have been tested for their anticancer potential in humans. Among the substances that have been tested are β-D-glucans or β-D-glucans linked to proteins. Moreover, the latter have demonstrated greater immunopotentiation activity than the free glucans. There are numerous clinical studies proving the cancer inhibitory effects of *Lentinus edodes* (Chihara et al., 1970; Hobbs, 2000; Zhang et al., 2011), *Grifola frondosa* (Dicks.: Fr.) Gray (Zhuang and Wasser, 2004; Boh and Berivic, 2007), *Schizophyllum commune* Fr.: Fr. (Hobbs, 2005; Zhang et al., 2013), *Ganoderma lucidum* (Lin 2009; Mahajna et al., 2010), *Trametes versicolor* (L.: Fr.) Lloyd (Hobbs, 2004), *Inonotus obliquus* (Mizuno et al., 1999; Balandykin and Zmitrovich, 2014), *Phellinus linteus* (Berk. et M.A. Curt.) Teng (Hsieh et al., 2013), *Flammulina velutipes* (W.Curt.: Fr.) Singer (Maruyama and Ikekawa, 2007), *Hypsizygus marmoreus* (Peck) Bigel. (Matsuzawa, 2006), *Ophiocordyceps* (=*Cordyceps*) *sinensis* (Berk.) G.H. Sung et al. (Holliday and Cleaver, 2008), *Agaricus brasiliensis* S. Wasser et al. (=*A. blazei* sensu Heinem.) (Wasser et al., 2002, 2005), *Tremella mesenterica* Retz.: Fr (Lo and Wasser, 2011; Lachter et al., 2012). Mushroom immunoceuticals act mainly by elevating the host immune system. This process includes activation of dendritic cells, NK cells, T cells, macrophages, and production of cytokines. Several MM products, mainly polysaccharides and especially β-glucans, were developed with clinical and commercial purposes: Krestin (PSK) and PSP (polysaccharide peptide) from *Trametes versicolor*; Lentinan, isolated from *Lentinus edodes*; Schizopyllan (Sonifilan, Sizofiran, or SPG) from *Schizophyllum commune*; Befungin from *Inonotus obliquus*; D-fraction from *Grifola frondosa*; GLPS polysaccharide fraction from *Ganoderma lucidum*; active hexose correlated compound (AHCC), and many others.

Other mushroom compounds of therapeutic interest are the secondary metabolites and especially some low-molecular-weight compounds such as lectins, lactones, terpenoids, alkaloids, antibiotics, and metal chelating agents, which are also important for the immune function of the organism. Low molecular sizes help them to penetrate through the cell membrane. MM also contain a number of enzymes such as laccase, superoxide dismutase, glucose oxidase, and peroxidase. It has been shown that enzyme therapy plays an important role in cancer treatment, preventing oxidative stress and inhibiting cell growth (Zaidman et al., 2005; Wasser, 2010).

It has been documented that fungi produce a huge number of biologically active compounds that not only stimulate the immune system but also modulate specific cellular responses by interfering in particular transduction pathways. For instance, the caffeic acid phenethyl ester (CAPE), which specifically inhibits the DNA binding of NF-κB and has shown some promising results in human breast cancer MCF-7 cells, was found to be produced by *Agaricus bisporus* (J. Lge) Imbach, *Marasmius oreades* (Bolt.) Fr., *Lentinus edodes*, and *Phellinus linteus.* Also, a methanol extract of *Fomes fomentarius* was reported to inhibit iNOS and COX expression due to the downregulation of NF-κB binding activity to DNA. Panepoxydone, a compound isolated from *Panus* spp. but also found in *Lentinus crinitus*, interferes with the NF-κB mediated signal by inhibiting the phosphorylation of IκBα. These reports demonstrate the fact that such substances can be used as molecular targets in malignant cells in order to combat cancer. Many fungal species have already been reported to produce various metabolites

capable of modulating different intracellular pathways thus playing an essential role in cancer treatment (Bresinsky and Besl, 1990; Zaidman et al., 2005; Chang, 2006; Yassin et al., 2008; Petrova et al., 2009; Wasser, 2010; Chang and Wasser, 2012; Chen et al., 2012; Chilton, 2016).

22.4 Medicinal Mushroom Dietary Supplements

MMs produce beneficial effects not only as drugs but also as a novel class of products known by a variety of names: DSs, functional foods, nutraceuticals, mycopharmaceuticals, and designer foods including prebiotics, which produce healthy benefits through everyday use. A group of Chinese researches published an article in *Nature Communications* pertaining to their research about the influence of *Ganoderma lucidum* on reducing obesity and modulating the composition of the gut microbiota (Chen et al., 2012).

DSs are not strictly used as pharmaceutical products; they also produce healthy effects through everyday use as part of a healthy diet.

Several types of MM DS products are available on the market today.

1) Artificially cultivated fruit body powders, hot water or alcohol extracts of these fruit bodies;
2) Dried and pulverized preparations of the combined substrate, mycelium, and mushroom primordia;
3) Biomass or extracts of mycelium harvested from submerged liquid culture grown in a fermentation tank or bioreactor;
4) Naturally grown, dried mushroom fruit bodies in the form of capsules or tablets.
5) Spores and their extracts (Chang and Wasser, 2012; Lindequist, 2013).

The increased interest in traditional remedies for various physiological disorders and the recognition of numerous biological activities of mushroom products have led to the coining of the term "mushroom nutriceuticals," which should not be confused with nutraceuticals, functional foods, and pharmaceuticals. A mushroom nutriceutical is a refined, or partially refined, extract, or dried biomass from either mycelium or the fruiting body of a mushroom, which is consumed in the form of capsules or tablets as a DS (not a food) and has potentially therapeutic applications. Regular intake may enhance the immune response of the human body thereby increasing resistance to disease and, in some cases, causing regression of the disease state. Thus, acting as immunopotentiators, MM preparations modify host biological responses (also known as BRMs).

There is no doubt that MM-based products can serve as superior DS. The market of DS from mushrooms is rapidly expanding and is valued at more than 18 billion US dollars (representing 10% of the general market of dietary supplements). Every year, data are collected as new evidence on the beneficial effects from DS made from MMs (Chang and Wasser, 2012). A new product for dementia (especially for Alzheimer's disease) based on a proprietary standardized extract that contains hericenones and amyloban (both from *Hericium erinaceus* – Lion's Mane mushroom) currently exists on the market. The value of DSs derived from the representatives of the genera *Ganoderma*, *Ophiocordyceps*, and *Cordyceps* is more than 4.0 billion USD per year.

22.4.1 Safety and Standardization of Medicinal Mushroom DSs

In this section, I would like to bring your attention only to the most important unsolved problems in studying DSs from MMs. Safety of the substances is considered a central feature of any regulation measure. The substances might have obvious or hidden beneficial actions or exploit

only a placebo effect, as long as the ideas of health are attached to them in the public's mind. However, their safety should be verified and proven as thoroughly as possible.

Drugs that affect body functions such as immune response, blood pressure, diuresis, and others are called pharmacodynamic agents. This is the way DSs are evaluated. Pharmacodynamics is the spectrum of biological responses produced by an intervention at a given time. The presence of therapeutic pharmacodynamic activity implies a lack of safety at a sufficiently high dose. This means that a mushroom preparation, like any other pharmacodynamic agent, cannot have pharmacological action without toxicological action. A completely safe agent would be without any activity whatsoever (Chang, 2006; Wasser, 2010; Chang and Wasser, 2012).

It is commonly believed that many plants and mushrooms can be considered safe because of their long history of usage. The Shiitake mushroom, for instance, was described in classical Chinese medicine (Shen Nong Ben Cao Jin, Compendium of Material Medica of the East Han dynasty) more than 2000 years ago (Mizuno, 1999).

However, safety to a pharmacologist is a relative concept, which is very different from the public notion of safety as an absolute concept. Here, I outline the reasons why we cannot take the safety of all mushroom-derived DSs for granted simply because they were used for many centuries in traditional human cultures:

1) Considering the historical perspective, "safety" in traditional terms is very different from that in modern times. Firstly, mortality patterns of developed societies today are very different from those of traditional ones. Death itself (the likelihood of dying at a certain age) had different kinetics in society. Secondly, traditional users rarely had the means to evaluate long-term or chronic toxicity of the agents. However, we do have cautionary instances of plants and mushrooms that have been used medicinally for centuries and recently proved to carry delayed toxic effects. One of the examples of an ill effect caused by mushroom is connected with *Paxillus involutus* (Batsch.: Fr.) Fr. Formerly it was widely used in Europe, and it is still taken as a food in many regions there. "*Paxillus* syndrome" was described only in 1971; in fact, this syndrome is an immunohemolytic anemia. A patient who has been eating *Paxillus* over a long period of time (sometimes years), on occasion may develop *Paxillus* syndrome in a short time period of one or two hours after consumption. An antigen of still unknown structure stimulates a severe immune shock resulting in such symptoms as diarrhea, subicterus, oliguria, anuria, hemoglobinuria, and renal pain (Bresinsky and Besl, 1990).
2) Many supposedly traditional mushroom products are now marketed in ways markedly different from those in the past. Today, larger amounts may typically be taken, or the material is used more frequently, or it is consumed in the form of enriched extracts, and it may be taken simultaneously with synthetic drugs. The user of Shiitake in old China, for instance, could not ingest as much active polysaccharide (Lentinan) as a modern user taking it in pure form extracted from Shiitake as a DS. Notably, 200 kg of fresh mushrooms are needed for extraction of 31 g of Lentinan (Chihara et al., 1970). This heightens the possibility of ill effects from traditionally "safe" mushrooms.
3) Also, many mushrooms or mushroom preparations traditionally taken as treatments for specific conditions are now often marketed for use as prophylactic agents. The idea of DSs, in many cases, implies that they are taken in the absence of any indicated conditions to prevent disturbances of health.

Because we lack strict data on the safety of many mushroom preparations, we could only make estimates on the basis of available scientific knowledge.

Chang (2006) suggested adopting the five "G" guidelines to enhance the quality of mushroom products. Although the text was originally oriented toward mushroom fruit bodies, it is still valid for other forms of mushroom products such as submerged culturing:

1) GLP (Good laboratory practice): A known mushroom strain must be used; the source and nature of the strain culture must be clearly documented and the culture should be properly maintained and preserved without contamination and degeneration.
2) GAP (Good agriculture practice): Growth conditions must be known; the substrate should be free of heavy metals and composed of consistent proportion of ingredients; the environmental conditions should include unpolluted air and good sanitary conditions of growth area. The product should be harvested at optimal maturity and free of diseases.
3) GMP (Good manufacturing practice): The parameters for the process must be known and maintained. The temperature, duration, and percentage of solvates used in extraction should be constantly monitored.
4) GPP (Good production practice): The following tests must be conducted: a chemical analysis of the products to determine organic components and heavy metal contents; a microbial analysis to determine if the type and level of micro-organisms present is within safe limits; and standardization of the formulation of the products.
5) GCP (Good clinical practice): Medical practitioners must conduct high-quality clinical trials including double-blind studies, which should be carried out to ensure standardization and allow appropriate dosage determinations and product formulation for the effective treatment of a particular health problem.

The fifth "G" is somewhat problematic to the developers of DSs. Once a clinical trial on humans is performed and bioactivity is confirmed, the product acquires a status of an unapproved drug. Until the product is approved, it is banned from the market because it contains an unapproved drug. The process of drug approval is extremely expensive and lasts several years. By the time the product is approved, the manufacturers may lose their business. This problem requires that only large pharmaceutical companies run clinical trials and those large companies do not engage in the research of traditional DSs. Another reason why DS developers do not wish to transform a health product into a medical product is because some health organizations, such as the FDA, do not approve a mixture of molecules, as in the case of mycelial or fruit body extracts, but only well defined, patentable molecules. On one hand, most DSs marketers will be just happy enough to sell their mushroom products without printed health claims. They will advertise them in foreign markets that allow health claims on the Internet or by word of mouth. On the other hand, perhaps a whole new industry, or even industries, will arise having greater economic value than those currently producing mushrooms for food.

The FDA has published guidance for the industry: Botanical Drug Products (www.fda.gov). This guidance explains when a botanical drug may be marketed under an over-the-counter (OTC) drug monograph and when FDA regulations require approval for marketing of a new drug application (NDA). In addition, this document provides sponsors with guidance on submitting investigational new drug applications (INDs) for botanical drug products, including those currently lawfully marketed as foods (including conventional foods and DSs) in the United States. Botanical products are finished, labeled products that contain vegetable matter as ingredients. A botanical product may be a food (including a dietary supplement), a drug (including a biological drug). The term botanicals includes plant materials, algae, and macroscopic fungi. It does not include fermentation products such as extracts made from submerged fermentation of mushroom biomass. Also, a botanical drug substance does not include a highly purified or chemically modified substance derived from natural sources. The authors are not aware of any clinical trials in the US with mushrooms as the botanical drug.

There are many problems in labeling medicinal mushroom products. This issue was discussed by Chilton (2016). Many MM products consist of mycelium propagated on grain,

what some call a biomass, since the grain is not separated from the final product. The overwhelming majority of these products are marketed and labeled as mushrooms. This is a classic case of mislabeling since there are no pure mushrooms in these products. Furthermore, the presence of the grain needs to be listed on the label as an ingredient since these biomass products are not 100% pure mycelium but new and novel products. The actual amount of mycelium in the biomass can be readily tested by ergosterol analysis (Hyde et al., 2010), and the grain residue can be measured by a starch test. If the grain is not listed as an ingredient of this mycelium biomass product, it could reasonably be considered an adulterant.

In the case of a blend of different MM stages, proper labeling would state the percentage of each stage that was included. One could not simply say mycelium, mushroom, and spores. Some biomass manufacturers claim mushroom inclusion due to the presence of mushroom primordia. Primordia are not actual mushrooms (fruit bodies), but simply the initial mycelium mass that, over time, becomes a mushroom.

The FDA has already issued a statement in their Compliance Policy Guide, "Section 585.525: Mushroom Mycelium – Fitness for Food; Labeling." It states: "Any food in which mushroom mycelium is used should be labeled to state that fact. Labeling should not suggest or imply that the food contains mushrooms" (Wasson, 1968). Proper labeling of MM products is an issue that cannot be ignored. With five primary fungal ingredients in the marketplace – mushroom, pure mycelium, mycelium on grain biomass, sclerotium, and spore preparations – proper labeling is more essential than ever for manufacturers to know what they are selling (Chilton, 2016).

22.4.2 Submerged Culturing as the Best Technique for Obtaining Consistent and Safe Mushroom Products

Today, approximately 80% of mushroom products are taken from fruit bodies either collected in the wild or grown commercially. In both cases, the resulting products are considerably diverse and unpredictable. The quality of mushroom fruit bodies is strongly dependent on substrate composition and properties of its ingredients, and usually these are far from constant. This is explained by the fact that the main components for mushroom production are of available agricultural and forest origin such as cereal straw, corn stalks, horse or chicken manure, wood sawdust, and so on.

The cultivation of mushrooms for fruit body production is a long-term process, taking one to several months for the first fruiting bodies to appear, depending on species and substrate. By contrast, the growth of pure mushroom cultures in submerged conditions in a liquid culture medium allows one to accelerate the speed of growth and reduces its duration to several days. Optimization of culture medium composition and the physicochemical conditions of growth allow regulation of mushroom metabolism to obtain a high yield of biomass and large amounts of specific substances of consonant composition (Anke, 1989; Boh and Berivic, 2007; Wasser, 2010; Lo and Wasser, 2011; Chang and Wasser, 2012).

22.5 Evidences, Challenges, and Unsolved Problems

On the one hand, MM science has made great progress in the last 30 years. A successful new branch of science (Medicinal Mushroom Science) has been widely recognized. New classes of MM drugs and different types of MM products have been developed. A unique journal in the field, the *International Journal of Medicinal Mushrooms* (Begell House, USA) was created. Every 2 years an International Medicinal Mushroom Conference is held; the next one (the ninth)

will take place in September 2017 in Palermo (Italy). A new International Society of Medicinal Mushrooms was established and registered in Canada in 2012.

Approximately 600 clinical trial studies on different illnesses using MMs have been published. More than 50,000 scientific papers have been published and approximately 15,000 patents dedicated to the studies of different aspects of MMs were received. From 2005, around 250–350 patents were registered each year for *Ganoderma lucidum* alone (Boh, 2013). Taiwanese scientists received more than 100 patents on one species from the genus *Antrodia*.

On the other hand, there are many unsolved serious problems in the future of MM science development, which in turn can also affect the continuation of this discipline in the twenty-first century. Next, the most critically important problems in the continuing development of MM science are listed.

22.5.1 Taxonomy and Nomenclature of MMs

Many species of MMs are critically misunderstood. Without the correct scientific name of the MM from the onset, future investigations will have no validity. Together with classical taxonomical methods, DNA barcoding may be useful and helpful for the correct identification of MM species, including the study of type material and standardization of MM products.

One of the major cases of incorrect identification and subsequent problems caused by it is the situation around *Pleurotus florida*. This incorrect naming of the species had been repeated in mycological literature for many years. However, there is no mushroom with such name. The mushroom culture named *P. florida* that has been distributed throughout many mycological culture collections represents, in fact, *P. ostreatus.* Here is a short historical remark aimed to clarify the naming problem. In 1965, in Germany, Dr. Gerlind Eger published her study on an oyster mushroom that she received from Florida, USA (Reshetnikov et al., 2001; Van Griensven, 2009). The strain had been isolated by a person named Block and sent to Dr. Eger labeled as *P. ostreatus* (Jacq. ex Fr.) Quél. Dr. Eger fructificated the culture and saw that it did not look the same way as *P. ostreatus* fruiting bodies of German origin. She consulted Dr. AH Smith (Ann Arbor, Michigan, USA), who found some similarities of studied strains with *P. cornucopiae* Paul. ex Fr. Thus Dr. Eger named the mushroom *Pleurotus florida*, however, without any description of a new fungal species as it is required under nomenclature regulations. This was a crucial mistake, and an act against all taxonomy and nomenclature rules. Dr. Eger was a recognized expert in the field of mushroom fructification and fruit body regeneration, however, probably not so experienced in fungal nomenclature. Several fungal taxonomists protested against the name *P. florida* and declared the name invalid (*nomen nudum*). Dr. Eger soon realized this mistake and in her next publication used the name "The fungus *Pleurotus* from Florida." Contemporaneously with this taxonomic mistake, the cultures of the fungus named *P. florida* were distributed over many fungal culture collections and received by many mushroom growers, due to great characteristics – good mycelium growth and fruiting body production (also at higher temperature), along with other advantageous features. Currently, the correct name of this mushroom from Florida is *P. ostreatus* (Jacq.: Fr.) Quél. cv. Florida (cv. means cultivar). Sometimes the abbreviation cv. is replaced by f. sp. (*forma specialis*) or the fungus is simply considered as an albino form of *P. ostreatus* (Sasek, 2015).

Here is another example. There is mounting evidence demonstrating that most samples previously reported as Lingzhi or Reishi (*Ganoderma lucidum*) in many pharmacological studies were mistakenly identified. *Ganoderma lucidum* presents a taxon-Linnean or species-complex future subdivision which requires caution (Wasser et al., 2006). Publications, patents, and products are also at risk. Through the years, at least 166 laccate *Ganoderma* species have been

described worldwide (Moncalvo and Rivarden, 1997). For instance, it is not known what are the taxonomic positions of so-called medicinal Blue, Red, or White Lingzhi.

Also, it is important to note that Cao with his colleagues (2012) published a paper in which they claimed that the so-called *G. lucidum* from China is quite different from *G. lucidum* found and described from Europe. The authors introduced a new species for science, *G. lingzhi* Sheng H. Wu, Y. Cao et Y.C. Dai. This brought more problems and confusion. Who knows now which *Ganoderma* species is medicinal? Which species of Lingzhi is the Chinese national mushroom *G. lucidum* or *G. lingzhi*? Probably nobody can answer this question for sure. Yang and Feng (2013) published a special mini-review dedicated to this problem called "What is the Chinese 'Lingzhi'?" Molecular phylogenetic analyses based on the ITS and 25S ribosomal DNA sequences indicated that most of the collections called *G. lucidum* in East Asia were not conspecific with *G. lucidum* found in Europe (Yang and Feng 2013).

Moreover, in 2012, in *Nature Communications*, a group of scientists published a paper dedicated to genome sequences of the model medicinal mushroom *G. lucidum* (Chen et al., 2012). Authors reported *G. lucidum* 43.3-mB genome, encoding 16,113 predicted genes, obtained using next-generation sequencing and optical mapping approaches. However, this very important publication didn't solve the problem of the *G. lucidum* species complex because authors studied one dikaryotic strain, CGMCC5.0026, belonging to the *G. lucidum* Asian group from China, which was claimed as the new species for science – *G. lingzhi.*

Another example of mistaken identity was revealed for *Agaricus blazei*, well known in literature as a MM. However, *A. blazei* is a North American endemic species described only from three localities and it does not exist in culture; therefore, it cannot be listed as a MM. Two concepts of *A. blazei* exist: *A. blazei* sensu Murrill, reported from three localities in the USA, and *A. blazei* sensu Heinem., reported from Brazil and cultivated in Japan (Wasser et al., 2002; 2005). We studied type material of *A. blazei* sensu Murrill, *A. blazei* sensu Heinem., *A. subrufescus* from the New York Botanical Garden (NY), and other species of this group, as well as cultivated strains from different countries, and material from nature in Brazil. It was revealed that *A. blazei* sensu Murrill and *A. blazei* sensu Heinem., represent two different species. *Agaricus blazei* sensu Murrill differs from *A. blazei* sensu Heinem., in size, shape of fruit bodies, pileal surface, type of pileal covering, presence of cheilocystidia, and spore size. It was determined that the widely cultivated culinary-medicinal mushroom known as *A. blazei* had nothing in common with *A. blazei* described by Murrill from the USA, and therefore a new species for science was described as *A. brasiliensis* (Wasser et al., 2002; 2005). *Agaricus blazei* is no longer known as a culinary-medicinal mushroom. Later, using morphological data along with molecular and biological data, the differences between *A. blazei* and *A. brasiliensis* were proven (Wasser et al., 2005). Several articles have been published by Wasser with co-authors (Wasser et al., 2002, 2005; Wasser, 2007) and Kerrigan (2005) in order to clarify the distinction between *A. brasiliensis*, *A. subrufescens*, *A. fiardii*, *A. praemagniceps*, and *A. blazei.* These species are now classified by a number of distinct morphological, molecular, and biological characteristics, and also different geographical distribution. The misclassification of *A. blazei* caused many problems in MMs science but has finally been corrected. I would like to bring your attention to the incorrect use of the name *A. blazei* for culinary-medicinal Royal Sun *Agaricus* or the Himematsutake mushroom. *Agaricus blazei* is not a medicinal mushroom; this species does not exist in culture and is known only from three localities in the USA! The same is true for *A. subrufescens.*

22.5.2 The Study of Culinary-Medicinal Mushrooms in Pure Culture

More attention must be paid to the study of culinary-medicinal mushrooms in pure culture. The study of cultures is necessary to provide stability and continuity in scientific work. The

teleomorph stage is the most essential criterion for the identification of cultures, but every so often MMs do not produce fruit bodies in pure culture. Vegetative mycelia of MMs in pure culture have received too little attention in mycological literature, although many species of MMs cannot be identified correctly without the study of vegetative mycelia. Vegetative mycelia of MM cultures represent complexes of branched hyphae, which differ only within narrow limits of width, length, number of nuclei, thickness of cell wall, and branching. The accumulation of information on an increasing number of studies of vegetative mycelia of MM species provides new material for the study and comparison of morphological characteristics of mycelia and for the estimation of their potential use for taxonomic purposes and purity control in biotechnological processes (Buchalo et al., 2009; Wasser, 2010; Chang and Wasser, 2012). Because there are no type strains of MMs, we need to choose correctly identified type strains of many species of MMs. We need to organize a world culture collection of culinary-medicinal mushrooms with depository activity following patent procedures according to the Budapest Treaty. This issue must be discussed with the World Federation of Culture Collections (Wasser, 2010; Chang and Wasser, 2012).

22.5.3 Medicinal Mushroom DS Problems

Recently, there has been growing popularity in developing mushroom biomass or different extracts as DS or functional foods and novel prebiotics (non-digestive β-glucans). Significant questions arise with establishing DS and MM products including their safety, standardization, regulation, efficacy, and mechanism of action.

Unfortunately, standardization of DS from MMs around the world is still in its early stages, including an insufficient understanding of DS bioactive effects. We don't have internationally recognized standards and protocols for the production and testing of MM products. Only proper standards and protocols can guarantee product quality. Without consistency in the quality of MM products, commercially available MM preparations will be dramatically different and vary enormously in composition and effectivity. It is not known whether bioactive effects are caused by a single component or are the result of a synergistic impact of several ingredients. There are insufficient data to determine which components demonstrate better effects – mushroom fruiting bodies or submerged mycelia, powders, or extracts. Are simple dried fruiting bodies and mycelia powders as effective as hot water, alcoholic, or hydro-alcoholic extracts? If we choose between crude extracts and isolated fractions, which one is more effective and has a higher safety profile (some companies are selling fraction-like *Grifola frondosa* Maitake D-Fraction or GLPS fraction of *Ganoderma lucidum*)? Also, the role of low-molecular-weight compounds in MM extracts is still unclear.

What is more effective – the combination of components containing biomass or extracts of 2–10 different mushroom species in one pill or just a single mushroom in one pill? How can one assess the effectiveness of different mushroom products when blending in many species in a single product ("shotgun" approach)? Since mushroom products can be cytokine stimulants, from what age can they be safely prescribed for young children taking into account that their immune systems are not yet mature?

Data regarding the dosages used is a very controversial issue. The suggested dosages vary a lot due to different forms and formulations. Numerous clinical trials have established that six capsules (3 capsules twice per day, or 2 capsules 3 times per day) 500–1000 mg each (biomass or extracts) is the accepted dosage of MM preparations. According to TCM, the standard dose of the dried fruiting bodies or biomass of MMs per day in different forms (tablets, capsules, liquid extracts, etc.) must be equivalent to about 100–150 g of fresh mushroom material.

What dosages are safe and effective during pregnancy and nursing? The absence of elaborated standards for the recommended use of MM DSs, including precise doses and duration of administration, needs very serious investigation. Some research show that too high dosages could lead to immune suppression; on the other hand, too low dosages might not trigger an immune response.

Furthermore, major problems associated with mushroom-based DSs are due to their wide variability, the current lack of standards for production, and the lack of testing protocols necessary to guarantee product quality. The active ingredients of many present-day commercial mushroom products have not been indicated.

The result of the current lack of standards for production and product quality of commercially available mushroom preparations from the same species and strains of mushrooms is the fact that they all are dramatically different in composition and effectivity. Adulteration of MM products with similar or spurious species (for instance, different *Ganoderma* species instead of *G. lucidum*; *Stereum* species replacing *Trametes versicolor*; different *Cordyceps* species, and even different anamorphic species of *Cordyceps* in place of *Ophiocordyceps sinensis*) is very common.

Difficulties are found in producing pure β-glucans for the market (90–95% of β-glucan on the market is considered counterfeit and adulterated). Adulteration has led to a number of adverse effects resulting in nephropathy, acute hepatitis, coma, and fever (McKenna et al., 2002; Wasser, 2010; Chang and Wasser, 2012).

We still have not solved the problems concerning the safety of several well-known MM products. For example, on the basis of phase I/II trials on the influence of *Grifola frondosa* polysaccharide extract in breast cancer patients, it was concluded that the Maitake mushroom produces more complex effects than presumed and may depress as well as enhance immune function (Deng et al., 2009).

What are the benefits of fresh mushroom consumption? The consumption of fresh mushrooms was found to increase, for example, anti-β-glucan antibodies in the serum of humans. The Ohno group from Japan also suggested that consumption of fresh mushrooms would provide better defense against pathogenic fungi (Ishibashi et al., 2005).

However, the information on inter-crossing or interaction of MM DSs with many commonly used drugs is still lacking.

22.6 Medicinal Mushroom Natural Products as an Unclaimed Source for Drug Discovery

The development of real immunomodulating and anticancer drugs from MM polysaccharides (e.g., Lentinan, Schizophyllan, and Krestin) was hampered by the fact that high-molecular-weight compounds are used in these drugs (Zhang et al., 2011, 2013). All MM drugs were developed from high-molecular-weight polysaccharides ranging from 100,000 to 0.5 million Da. These compounds cannot be synthesized; therefore, their production is restricted to extraction from fruit bodies, cultured mycelium, or cultured broth. Such an approach imposes high-market prices. Today, science should concentrate on the beneficial medicinal effects of low-weight molecular compounds produced by MMs, that is, low-molecular-weight secondary metabolites targeting processes such as apoptosis, angiogenesis, metastasis, cell cycle regulation, and signal transduction cascades (Zaidman et al., 2005). Western pharmaceutical companies are more interested in relatively easily synthesized compounds that can be produced for markets.

Historically, the majority of new drugs have been generated from natural products (secondary metabolites). MMs are an unclaimed source for drug discovery. By 1990, about 80% of drugs

were either natural products or analogs inspired by them. "Blockbuster drugs" like antibiotics (penicillin, tetracycline, and erythromycin), antiparasitics (avermectin), anti-malarials (quinine, artemisinin), lipid control agents (lovastatin and its analogs), immunosuppressants for organ transplants (cyclosporin, rapamycins), and anticancer drugs (taxol, doxorubicin) revolutionized medicine (Li and Vederas, 2009). Many of the abovementioned drugs were discovered from components found in fungi.

Modern pharmaceutical trends in preventing cancer include the development of new drugs with (1) growth-factor inhibitors of cancer cells (such as herceptin, erbitux, and terceva). They block a cancer cell's link to critical proteins that help it divide and grow. (2) Hormone blockers (drugs such as tamoxifen) keep cells from dividing by binding to estrogen receptors, which are over-expressed in some tumor cells, and (3) signal blockers working inside a cell. These drugs interrupt communication among enzymes that regulate growth and development, and (4) angiogenesis inhibitors, for example, avastin, that was the first drug to inhibit the formation of new blood vessels around cancer cells, starving them of nourishment (Ammerpohl et al., 2010). About 860 cancer drugs are being tested on humans. This number is more than twice the number of experimental drugs for heart disease and stroke combined, nearly twice as many as for AIDS and all other infectious diseases combined, and nearly twice as many as for Alzheimer's and all other neurological diseases combined (Pollack, 2009). Cancer drugs have been the biggest category of drugs in terms of sales worldwide since 2006 and in the US since 2008, according to market research by IMS Health. Today, drug companies see a future in treating cancer. The world's largest pharmaceutical company (Pfizer), for example, was focused on cardiovascular drugs, the cholesterol-lowering buster Lipitor (Endo, 2004), and the blood pressure reduction pill Norvasc (Pollack, 2009). Recently, Pfizer hired about 1,000 researchers for an all-out effort to develop drugs for treating cancer, a disease the company once largely ignored. Pfizer has now scaled back on cardiovascular research and has made cancer drugs one of its six focus areas. About 20% of Pfizer's more than $7 billion budget for research and development is spent on cancer research, and 22 out of roughly 100 drugs being tested are anticancer drugs (Pollack, 2009).

Progress in research of MMs must include genomics, proteomics, metabolomics, and systems pharmacology. Studying molecular mechanisms to determine the medicinal effects of MMs should be the focus of new investigations using modern methods in the above approaches.

Another important source for substances of therapeutic interest can be found in the pool of secondary metabolites produced by MMs. These substances can be classified according to five main metabolic sources (Zaidman et al., 2005): amino acid-derived pathways; the shikimic acid pathway for the biosynthesis of aromatic amino acids; acetate-malonate pathway from acetyl coenzyme A; the mevalonic acid pathway from acetyl coenzyme A, which functions in primary metabolism for the synthesis of sterols; and polysaccharides and peptidopolysaccharides. The polyketide and the mevalonic acid pathways are most often involved, and they produce a greater variety of compounds than the other pathways.

Every effort should be put into finding new sources for anticancer drugs using low-molecular-weight secondary metabolites from MMs that can inhibit or trigger specific responses, that is, activating or inhibiting the NF-κB, inhibiting proteins and especially tyrosine kinases, aromatase and sulfatase, matrix metalloproteinases, cyclooxygenases, DNA topoisomerases and DNA polymerase, anti-angiogenic substances, and so on (Zaidman et al., 2005; Yassin et al., 2008; Petrova et al., 2009).

Fungal low-molecular-weight compounds directly influencing NF-κb inhibitory effects are: CAPE, cordycepin, panepoxydone, and cycloepoxydon. Low-molecular-weight CAPE produced by, for example, *Phellinus linteus* and *Marasmius oreades*, show specific cytotoxicity

against tumor cells, along with NF-κb inhibitor activity, and can be candidates for antitumor drugs, especially against breast cancer (Petrova et al., 2009).

Pharmaceutical companies involved in drug discovery need new sources of natural products. MMs are the best-unclaimed gift of nature that in a short amount of time can be used for the production of new pharmaceuticals. Here the author presents the Drug Discovery Pathway, which was specifically designed for the development of medicinal mushroom pharmaceuticals. This pathway includes nine steps:

1) Mushroom cultivation and biomass production
2) Biomass extraction
3) Screening of mushroom extracts
4) Effect of selected extracts on a target of interest
5) Chemical fractionation of selected extracts
6) Elucidation of active fractions (compounds) mechanism of action and potency
7) Effect on animal models
8) Preclinical drug development
9) Clinical drug development (Wasser, 2010)

22.7 Unsolved Problems in the Study of Structural Characteristics, Isolation Process, Receptor-Mediated Mechanism and Antitumor Activity of MM β-Glucans

The success of β-glucans and other mushroom carbohydrate polymers' application requires active research in addressing the structure-activity relationship of mushroom carbohydrate polymers, especially in terms of molecular conformation and receptor-mediated mechanisms (Chen and Seviour, 2007; Lee and Kim, 2014). Clarification of water solubility, size of molecules, molecular weight, structure, and molecular mechanisms of β-glucan action takes into consideration that not all β-glucans contained in MMs exhibit pharmaceutical activity (Ohno, 2005; Chen and Seviour, 2007).

The role of molecular weight in the pharmaceutical activity of β-glucans is still not known. The problem of effectivity of high-molecular-weight β-glucans compared to low-molecular-weight β-glucans still exists. Data shows that scleroglucan high-molecular-weight preparations are most effective (Ohno, 2005). But, on the other hand, only low-molecular-weight Lentinan has higher antitumor activity (Chihara et al., 1970; Zhang et al., 2007). The different reactivity of β-glucans in each individual must be taken into account (anti-β-glucan titer and increments of the titer by β-glucan administration are different; reactivity of peripheral blood leucocytes to β-glucan is significantly different in each individual; reactivity to β-glucans is, for example, significantly different in various strains of mice) (Ohno, 2005; Chen and Seviour, 2007; Zhang et al., 2007).

Solubility in water is one of the most important characteristics of β-glucans. It is still not known what are the major factors affecting the solubility and pharmaceutical activity of β-glucans: molecular weight, length of side chain, number of side chains on the main chain, ratios of (1,4), (1,6), and (1,3) – linkages; also, ionization by acid must be considered (Chen and Seviour, 2007; Zhang et al., 2007; Wasser, 2010). Soluble β-glucans appear to be stronger immunostimulators than insoluble β-glucans. The reasons for this are not totally clear. The exact mechanism of intestinal absorption of orally administrated β-glucan remains unknown (nonspecific intestinal absorption; passage of β-glucans through the gap junction in the intestinal epithelial membrane; absorption through intestinal M cells; absorption after binding with Toll-like receptor proteins on the intestinal lumen, and dendritic cell probing) (Miller et al., 2007;

Pamer, 2007). It is possible that orally administered insoluble β-glucans are subsequently degraded into smaller bioactive oligomers after ingestion (Lehmann and Kunze, 2000).

The differences between plant β-glucans (Tada et al., 2008; Tiwari and Cummins, 2009), yeasts β-glucans (Liu et al., 2009), and β-glucans from MMs (Ohno, 2005; Chen and Seviour, 2007; Zhang et al., 2007; Wasser, 2010; Lee and Kim, 2014) must be clarified. What is the difference in structure, solubility, and biological activity? For example, the structure of cereal β-glucan is essentially β-1,3 and β-1,4-linkages, not β-1,6-linkages. In addition, plant β-glucans are linear, not branched. Usually, the molecular weights of plant β-glucans are smaller than those of MM β-glucans. Biological activity has not been fully examined in the case of plant β-glucans. Usually, yeast β-glucans are only partially water-soluble, and many MM β-glucans are water insoluble. Why do they have different biological activity? What are the key advantages of MM β-glucans compared, for example, to cereal β-glucans, or yeast β-glucans?

We know a lot about the function of receptor dectin-1 (dendritic-cell-associated C-type lectin-1) of β-glucans (Taylor et al., 2007; Harada and Ohno 2008; Lee and Kim, 2014). β-glucans have antifungal activity that is similar to their anticancer activities and is mediated by binding to dectin-1. It is still not known how β-glucans bind to this receptor. However, there is plenty of information about the function of receptor dectin-1 of β-glucans, but the function of receptor dectin-2 is still unclear (Graham and Brown, 2009; Lee and Kim, 2014).

Why do β-glucans have the triple-helix conformation, and does the triple-helix structure have some advantages over a single strand structure (Ohno, 2005; Chen and Seviour, 2007; Zhang et al., 2007; Wasser, 2010)? Unfortunately, we do not understand what structural features are best for inducing specific activities and, even more importantly, what the presence of hydrophyllic groups located on the outside surface of the helix means. We can see contradictory data in the literature on the biological activity of triple-helix and single-helix structures of the same β-glucan – for example, Schizophyllan (Ohno, 2005; Chen and Seviour, 2007; Zhang et al., 2007). We still don't know which one has stronger biological activity – the closed triple-helix or a partially opened triple-helix (Mizuno, 1999; Falch et al., 2000). β-glucans of some species form triple-helix aqueous solutions. However, in the presence of alkaline pH or DMSO, the triple helix converts rapidly to a single-stranded helix, which gradually reforms a triple helix at neutral pH levels. However, the form of the helix associated with more effective immunomodulatory and antitumor activities is unknown (Lee and Kim, 2014).

22.8 Medicinal Mushroom Clinical Studies

The popularity of MMs and the number of their applications in modern medicine are constantly increasing. However, despite the wide acceptance of MMs, most of the population is not sufficiently informed in terms of health benefits, clinical efficacy, and the safety of medicinal mushroom usage. Unlike synthetic drugs, most of the products derived from MMs have not yet undergone rigorous evaluation based on rules and protocols of evidence-based medicine. Therefore, clinical studies are a highly important step in bringing new and effective drugs developed from MMs to the consumer at large.

Each clinical study involves research on human volunteers (healthy or with specific diseases/conditions) aimed to provide investigators with novel medical knowledge. There are two main types of clinical studies: clinical trials and observational studies.

22.8.1 Clinical Trials

Clinical trials represent investigations that are done in the framework of clinical research. These studies include tests and experiments carried out on groups of human participants and

are designed in order to supply researchers with new information and give answers to specific questions raised by scientists about biomedical or behavioral interventions. Participants are exposed to specific interventions according to the experiment's protocols or research plans created by the investigators. These interventions may include use of medical products such as drugs, novel vaccines, DSs; tests of newly developed medical devices and procedures; and even changes to participants' behavior, such as diet, and so on. The main idea behind the trial is to assess both safety and efficacy of the drug, device, procedure, and so on, on human beings by comparing results of participants exposed to test interventions with a group receiving control treatment. It is important to note that patients in both groups should be enrolled, treated, and followed over the same period of time. Studies in which test and control groups are exposed to treatment and followed over different time periods, experiments involving species other than humans, or studies that are carried out *in vitro* on tissues, cells, biological substances, and so on, obtained from humans are not accepted as clinical trials (Meinert and Tonascia, 1986).

The whole clinical trial should be designed, executed, and analyzed in such a manner to maximally ensure the statistical significance, scientific validity, and reproducibility of the obtained results. Ideally, the trial should be randomized (when test subjects are randomly allocated to different groups in the study), double-blind (in which both researchers and participants do not know the exact group each participant is assigned to) in contrast with single-blind or open-label trials (when either researchers alone or both researchers and participants are familiar with the type of treatment each subject is receiving), and placebo-controlled (when the trial has a control group that receives a sham treatment). However, in some cases, it is not possible to achieve this "gold standard."

Clinical trials may vary on the criteria of the outcome measurements, observation period, size, and cost. They can be carried out in a single research facility or present a collaboration of multiple facilities situated in different countries all over the world.

All medicinal substances before undergoing clinical trials are thoroughly investigated and tested *in vitro* and *in vivo* on animals to ensure their safety and efficacy. Only after such preliminary studies can the substance receive health authority/ethics committee approval to conduct a clinical trial so that the risk/benefit ratio of future studies is considered acceptable.

22.8.2 Observational Studies

Another clinical study design is observational studies. In contrast to clinical trials, participants in observational studies are not assigned to specific interventions by the investigators, although participants may receive some interventions or procedures as part of their routine medical care. In situations when it is not possible to conduct a valid clinical trial because of the objective limitations, including ethical aspects, observational studies may be the next best method to answer questions raised by the researchers. A well-designed observational study is capable of providing scientists with objective and statistically significant information, the quality of which will not be much far behind a randomized controlled trial. Cohort studies and case-control studies comprise the two main types of observational studies that have been used in medical science in order to establish a relationship between different diseases and exposures.

An example of an observational study may be the cohort study in which investigators follow for some significant period of time a group of smoking adults to find more data on the relationship between smoking habit and risks of developing different types of respiratory diseases.

A simplified table of the hierarchy of evidence from the Center for Evidence-Based Medicine (CEBM) is presented in Table 22.1.

One can see from the table that randomized controlled trials along with systematic reviews and meta-analyses of those trials hold the highest rank in the hierarchy. However, RCTs of

Table 22.1 Levels of evidence.

Level	Study Type
I	High-quality, multicenter or single-center, randomized controlled trials, or systematic review of randomized controlled trials
II	Individual cohort studies, low-quality randomized controlled trials, or systematic reviews of these studies
III	Individual case-control studies, or systematic reviews of case-control studies
IV	Case-series, poor quality cohort, and case-control studies
V	Expert opinions, case reports, or clinical examples, evidence based on physiology, bench research, etc.

lesser quality occupy a lower level of evidence (II), sharing it with high-quality observational studies, like individual cohort studies. Case reports, clinical examples, anecdotal evidence, and so on, present the lowest level of scientific evidence in modern medicine.

All general information on clinical studies, including defining the clinical study, that is, who conducts the research, the purpose and length of the study, reasons for conducting the study, who can participate in the study, and so on, can be found on the website of the US National Institutes of Health (www.ClinicalTrials.gov). This web site currently lists 206,158 studies with locations in all 50 states of the US and in 191 countries.

Clinical trials involving tests of different MM substances on patients diagnosed with specific diseases have been conducted all over the globe, especially in Asian countries. The findings from these clinical studies suggest that MM products may have multiple pharmacological activities (as their major active constituents are polysaccharides). These activities may be minor, moderate, or lacking, due to many factors such as inappropriate dosage regimen, difficulties in finding suitable biomarkers and end points, large inter-patient variability in responses to specific treatment, and unknown mode of action. Clinical studies of MMs may encounter great difficulties because specific MMs contain various active components; and therefore, there are problems in the standardization and quality control of their preparations. Pharmacokinetic studies also encounter multiple complications; therefore, a dose-response relationship is hard to establish. MMs themselves are always used as adaptogens and thus acceptable clinical biomarkers are often lacking. Further well-designed clinical studies are needed to identify the efficacy and safety of MM products in patients.

22.8.3 Regulation Issues

Different countries have created their own legislation and regulation systems for medicinal plants and substances derived from them in order to ensure quality control of these products and for the establishment of suitable standards for the consumer. These regulations introduce criteria for the assessment of safety, efficacy, and quality of DSs or medicinal substances and contain guidelines and requirements for licensing, dispensing, manufacturing, and labeling the products. The status of herbal or mushroom preparations may vary significantly depending on the country's laws and regulations. In Western and Eastern countries, herbal and mushroom medicines fall under different regulation procedures. In 1994, the Dietary Supplement Health and Education Act (DSHEA) was signed into law by the USA president. Under the definition of DSHEA, the dietary supplement presents a product that is used to supplement the regular diet and contains ingredients like vitamins, minerals, herbs, or other botanical, amino acids,

metabolites, different extracts, and so on, or various combinations of those ingredients. Most Western countries follow these principles and define plant/mushroom extracts as DSs, meaning that clinical studies (phase I–IV) are not mandatory for such products to be legally introduced to the market. However, China defines many herbs and some mushrooms as medicinal products or drugs and valid phase I–IV studies are necessary to receive an approval (Zhou et al., 2005).

Phase I–IV studies are required for new herbal and mushroom drugs before being claimed to possess therapeutic properties and to receive the approval of the State Food and Drug Administration of China. Clinical trials or clinical verifications of new drugs should be sanctioned by the Ministry of Public Health and provide sufficient data on toxicity, pharmacological properties, and efficacy. In phase I a new drug or herbal/mushroom preparation is tested on a small group of people to explore the general acceptance, evaluate its safety, determine a safe dosage range, and identify possible adverse effects. Phase II involves a larger group of test subjects in order to further evaluate the safety of a drug, while investigating its efficacy and determining the effective dosages; phase III is carried out to receive additional confirmation on effectiveness of the treatment, monitor most common adverse effects and supply researchers with information that will allow the drug/preparation to be used safely; phase IV studies are executed after the drug is commercially available, they are intended to gather information on rare side effects and possible complications connected with long-term usage, also they provide important information on the effects of the drug in various populations. Zhou et al. (2005) presented a scheme outlining the main objectives of different stages of clinical trials for herbal and mushroom medicines (Figure 22.1).

The creation of a unified international regulation code governing the market of DSs and medications derived from fungi and plants will significantly facilitate the availability of these products for common people all over the globe and will enable maintenance of a high level of quality and safety of these products.

Clinical studies on the effects of various MMs preparations on humans have been published in more than 400 papers and reports. Information about more than 40 clinical studies can be found on the web service of the US National Institutes of Health (www.ClinicalTrials.gov). The most promising data appear to be those indicating an inverse relationship between the use of MMs and breast, prostate, colorectal, cervical, ovarian, and gastric cancers, along with other conditions. Information on successful clinical trials that demonstrated positive effects of multiple MMs preparations is presented in Table 22.2. The table is based on the data compiled by Roupas et al. (2012) with additional data updating this information.

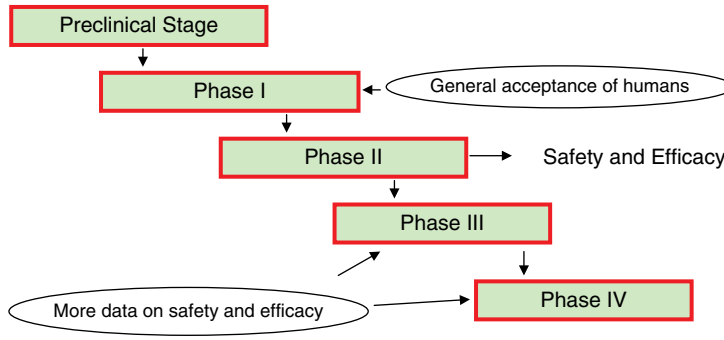

Figure 22.1 Objectives of clinical trials for herbal and mushroom medicines.

Table 22.2 Therapeutic activities of medicinal mushroom compounds and extracts evaluated in clinical studies.

Effect/Disease	Bioactive Ingredient or Extract	Species Name	Registered Positive Effects	Reference
Anticancer (breast)	Ergosterol Freeze-dried mycelial powder Spore powder	Unspecified variety *Trametes versicolor* *Ganoderma lucidum*	Increases serum 25 (OH) vitamin D2 levels A phase I, two center, dose escalation study done on women with breast cancer, who have already received chemo- and radiotherapy treatments, indicated increases in lymphocyte counts (doses of 6–9 g/day), increased functional activity of natural killer cells (dose 6 g/day), and increased numbers of $CD8^+$ T cells along with $CD19^+$ B cells, which were dose-related. Preparations of *T. versicolor* were well tolerated among the test subjects A pilot clinical trial involving 48 breast cancer patients with cancer-related fatigue, undergoing endocrine therapy demonstrated statistically significant improvements in the domains of physical well-being and fatigue subscale, along with less anxiety and depression, and better quality of life after administration of *G. lucidum* spore powder. The therapy showed minimum adverse effects and was well-tolerated	Furlanetto 2009[a]; Torkelson et al., 2012; Zhao et al., 2012
Anticancer (colorectal)	Polysaccharide K (PSK) (in adjunct with immunochemotherapy) Unspecified bioactive/ extract Unspecified bioactive/ extract Lentinan PSK	*Coriolus versicolor* CM-10 *Agaricus sylvaticus* *Ganoderma lucidum* *Lentinus edodes* *Trametes versicolor*	Stimulate both innate and adaptive immune pathways in curatively resected colorectal cancer Benefits in hematological and immunological parameters and reduction in glycemic levels Apoptosis (induced by increase in caspase-3 activity) and anti-inflammatory function in HT-29 in human carcinoma cells (no toxicity in HT-29 cells in doses <10 mg/ml) Multicenter clinical study demonstrated safety and efficacy of oral formulation of superfine dispersed Lentinan (SDL) for suppressing the adverse effects of chemotherapy as well as improving quality of life scores (QOL) in patients with advanced colorectal cancer tegafur/uracil plus PSK 5-year overall survival rate significantly higher in the PSK group ($p < 0.016$, $p < 0.056$, respectively)	Oba et al., 2009; Sakamoto et al., 2006; Fortes et al., 2009; Hong et al., 2004; Hazama et al., 2009; Ohwada et al., 2004

Anticancer (cervical, ovarian, endometrial)	Unspecified extract Schizophyllan	*Agaricus blazei* Murrill Kyowa (AbMK) *Schizophyllum commune*	Increased activity of natural killer cells along with improved chemotherapy side effects (e.g., appetite, alopecia, emotional stability and general weakness) in patients undergoing chemotherapy Randomized study involving 220 patients with Stage II or Stage III cervical cancer demonstrated significantly longer survival time among Stage II patients in Schizophyllan (SPG) group	Ahn et al., 2004; Okamura et al., 1986
Anticancer (gastric)	Lentinan (in combination with tegafur and cisplatin) Lentinan PSP	*Lentinus edodes* *Trametes versicolor* (Yun Zhi)	Significantly longer median survival (297 days vs 199 days) and greater one-year survival rate (49.1% v. 0%), along with improved total QOL score (especially appetite and the quality of sleep) in the Lentinan group compared to the control Meta-analysis of individual patient data from 5 trials (650 patients) showed that Lentinan in conjunction with chemotherapy significantly prolongs overall survival compared with chemotherapy alone Addition of PSP to radiotherapeutic or chemotherapeutic treatments can greatly improve the QOL and reduce chemotherapy-induced side effects, weakness, anorexia, vomiting, dryness of throat, spontaneous sweat, and pain symptoms	Nakano et al., 1999; Oba et al., 2009; Zhong et al., 2001
Anticancer (prostate)	Ethanol extract of whole mushroom Mushroom powder in the form of tablets	*Ganoderma lucidum* *Agaricus bisporus* (white button mushroom)	Dose extract 6 mg per day improve the total International Prostate Symptom Score (IPSS) of men with lower urinary tract symptoms via strong 5-alphal reductase inhibitory activity Phase I trial demonstrated that the therapy with white button mushroom powder appears to impact both prostate-specific antigen levels and modulate the biology of biochemically recurrent prostate cancer by decreasing immunosuppressive factors	Noguchi et al., 2008a, 2008b; Twardowski et al., 2015
Anticancer (pancreatic – advanced solid malignancy)	Irofulven (cytotoxin)	*Omphalotus olearius* Note: Not edible	Daily dose of 10.64 mg/m^2 as a 5 min i.v. infusion for 5 days every 4 weeks resulted in antitumor activity; and intermittent dosing schedules had positive preclinical antitumor effects	Eckhardt et al., 2000

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Table 22.2 (Continued)

Effect/Disease	Bioactive Ingredient or Extract	Species Name	Registered Positive Effects	Reference
Anticancer (lungs)	Ganopoly® (water-soluble polysaccharides)	*Ganoderma lucidum*	Open-label study involving 36 patients with advanced lung cancer demonstrated markedly modulated immune functions in some patients, suggesting that some subgroups of cancer patients might be responsive to Ganopoly® in combination with chemotherapy/radiotherapy	Gao et al., 2005
Anticancer (myeloma)	Mushroom extract AndoSan™ containing 82% of *Agaricus blazei* Murill, 14.7% of *Hericium erinaceus,* and 2.9% of *Grifola frondosa*	*Agaricus blazei* Murrill *Hericium erinaceus Grifola frondosa*	A number of immunomodulating effects were observed in the group of patients diagnosed with multiple myeloma undergoing stem cell mobilization followed by high dose chemotherapy and using AndoSan™ product. An increased percentage of Treg cells and plasmacytoid dendritic cells were found in the leukapheresis product harvested after stem cell mobilization. Also, a significant increase of serum levels of IL-1ra, IL-5, and IL-7 at the end of treatment was found in the AndoSan™ group. Whole genome microarray demonstrated increased expression of immunoglobulin genes, Killer Immunoglobulin Receptor genes (KIR), and HLA genes in the AndoSan group	Tangen et al., 2015
Anticancer (hepatocellular carcinoma)	Lentinan	*Lentinus edodes*	Combination of Lentinan with transcatheter arterial chemoembolization (TACE) and radiofrequency ablation (RFA) demonstrated significantly higher tumor necrosis rate (88.6%), compared to the TACE group (37.5%), RFA group (47.8%) and TACE/RFA group (60.3%); lower tumor recurrence rate (17.8%) with 45.8%, 34.7% and 29.0% in the TACE, RFA and TACE/RFA groups, accordingly. Finally, therapy involving Lentinan was beneficial in terms of increasing mean survival duration (28.2 months) A superfine dispersed Lentinan-containing supplementary food can significantly increase survival times in hepatocellular carcinoma patients. The rates of Lentinan-binding CD14+ monocytes could be a promising prognostic predictor. Long-term ingestion of superfine dispersed Lentinan increases its health benefits	Yang et al., 2008; Isoda et al., 2009

Anticancer (different conditions)	Ganopoly® (polysaccharides fractions)	*Ganoderma lucidum*	Increase in the mean plasma concentrations of interleukin (IL-2), IL-6, and interferon (IFN)-g, while the levels of IL-1 and tumor necrosis factor (TNF-a) were decreased. After 12-week treatment with Ganopoly, the mean absolute number of CD56 cells was significantly increased; PHA responses were also enhanced in most patients. It was demonstrated that Ganopoly® may enhance the immune function of patients with advanced-stage cancer. However, the immune response may vary in different subgroups of cancer patients. Thus, some patients may get better results from Ganopoly® treatment in combination with conventional chemotherapy/radiotherapy. Note: clinical evaluations of response and toxicity remained an open question	Gao et al., 2003
Anticancer (immuno-modulation)	Glucan	*Trametes versicolor*	Improved survival and immune function	Ramberg et al., 2003
Immuno-modulation: (post-menopausal breast cancer)	Polysaccharide extract	*Grifola frondosa* (Maitake mushroom)	Immunologically stimulatory and inhibitory measurable effects in peripheral blood of patients free of disease after 1st treatment	Deng et al., 2009
Immuno-modulation: (healthy volunteers)	AndoSan™ Dried fruit bodies	*Agaricus blazei* Murrill (AbM) (Himematsutake) 82% *Hericium erinaceus* (Yamabushitake) 14.7% *Grifola frondosa* 2.9% *Lentinula edodes* Shiitake	*In vivo* there was a significant reduction in levels of IL-1-b (97%), TNF-a (84%), IL-17 (50%), and IL-2 (46%). Discrepancy in results associated with antioxidant activity of AbM *in vivo* and limited absorption of its large β-glucans across the intestinal mucosa to the reticuloendothelial system and blood 52 healthy males and females (21–41 years old), involved in a 4-week parallel-group study, improved immunity, which was demonstrated by improved cell proliferation and activation, and increased sIgA production. The reduction in CRP suggested lower inflammation. The therapy resulted in increased interleukin (IL)-4, IL-10, tumor necrosis factor (TNF)-α, and IL-1α levels, a decreased macrophage inflammatory protein 1α/chemokine C-C ligand 3 (MIP-1α/CCL3) level	Johnson et al. 2009; Dai et al., 2015

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Table 22.2 (Continued)

Effect/Disease	Bioactive Ingredient or Extract	Species Name	Registered Positive Effects	Reference
Immuno-modulation (mild hypercholesterolemia)	α-glucans	*Agaricus bisporus*	Consumption of fruit juice enriched with 5 g glucans/day lowered lipopolysaccharide-induced TNF-α production by 69%. No effects on IL-1b and IL-6 and decreased production of IL-12 and IL-10 was observed	Volman et al., 2010a
Diabetes (type II)	AbM extract (in combination with metformin and gliclazide) Ganopoly® (polysaccharide fractions)	*Agaricus blazei* Murrill (AbM) *Ganoderma lucidum*	Improve insulin resistance potentially by the mechanism that caused an increase in adiponectin concentration after taking the extract for 12 weeks Phase I/II trial reported significant changes in mean FPG and PPG levels which paralleled the changes in mean HbA_{1c} levels after 12 weeks of treatment. PPG value in patients treated with Ganopoly® decreased from 13.6 mmol/L to 11.8 mmol/L. Overall, the study demonstrated that Ganopoly® therapy was efficacious in lowering blood glucose concentrations and was well tolerated	Hsu et al., 2007; Gao et al., 2004a
Diabetic nephropathy	JinShuiBao capsules (fermented mushroom mycelia, Cs-4 strain)	*Cordyceps sinensis*	JinShuiBao capsules combined with alprostadil injection demonstrated good therapeutic effect in patients with early diabetic nephropathy showing significant decrease in blood urea nitrogen and serum creatinine levels, along with urine albumin excretion rate	Lu and Wu, 2013
Cardiovascular diseases	Unspecified bioactive/ extract Protein-bound Polysaccharides (A-PBP and L-PBP) Ganopoly® (polysaccharide fractions)	*Pleurotus ostreatus* (oyster mushroom) *Agaricus blazei* *Lentinus edodes* *Ganoderma lucidum*	Significant reduction in systolic and diastolic blood pressure, blood glucose, total cholesterol and triglycerides Weight-controlling and hypolipidemic effect via a mechanism involving absorption of cholesterol Double-blind, randomized, multi-centered phase I/II studies on 160 patients diagnosed with coronary heart disease (CHD) indicated that Ganopoly® treatment significantly improved primary symptoms (chest pain, palpitation, angina pectoris, and shortness of breath) of CHD compared to the control group. Significant decrease in the percentage of abnormal ECG, decrease in blood pressure, and serum cholesterol levels were observed after 12 weeks of Ganopoly® therapy. The treatment with Ganopoly® was well tolerated	Khatun et al., 2007; Kweon et al., 2002; Gao et al., 2004b

Hypolipidemic	JinShuiBao capsules (fermented mushroom mycelia, Cs-4 strain)	*Cordyceps sinensis*	Randomized, double-blind, placebo-controlled study involving 273 patients with hyperlipidemia (elevated cholesterol, elevated triglycerides or both) showed that JinShuiBao lowered total cholesterol and triglycerides levels, and increased high density lipoprotein levels in the test group	Geng, 1995
Brain health and cognition	Dried and powdered fruiting bodies Dilinoleoyl-phosphatidylethanolamine (DLPE) Hericenones C to H Erinacines A to I Powdered fruiting bodies added to cookies Ganopoly® (polysaccharide fractions)	*Hericium erinaceus* (Yamabushitake) *Ganoderma lucidum*	Double-blind, parallel-group, placebo-controlled trial demonstrated significant increase in scores on cognitive function scales in men and women (aged 50–80 years old) diagnosed with mild cognitive impairment who were taking tablets containing *H. erinaceus* Protect against neuronal cell death caused by β-amyloid peptide (A β) toxicity, endoplasmic reticulum (ER) stress and oxidative stress. Improve the Functional Independence Measure (FIM) score or retard disease progression in patients with dementia Induce synthesis of nerve growth factor (NGF) (*in vitro* and *in vivo*) Randomized, double-blind, placebo-controlled trial conducted on 30 females for a period of over 4 weeks showed a significant decrease in CES-D and ICI scores in a group that was taking cookies with the mushroom. These results demonstrated that *H. erinaceus* has a possibility to reduce depression and anxiety, and also suggested a different mechanism from NGF-enhancing action of the mushroom Phase I/II randomized, double-blind, placebo-controlled parallel study involving 123 patients with neurasthenia, who received 1,800 mg of either Ganopoly® or placebo during 8 weeks, showed significant reduction in CGI severity score and sense of fatigue, increase in well-being score, and some other parameters in the treatment group compared to the placebo. The therapy was well tolerated	Mori et al., 2000; Kawagishi and Zhuang, 2008; Nagano et al., 2010; Tang et al., 2005

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Table 22.2 (Continued)

Effect/Disease	Bioactive Ingredient or Extract	Species Name	Registered Positive Effects	Reference
Antiviral (hepatitis B)	Ganopoly®	*Agarius blazei* Murrill (AbM) *Ganoderma lucidum*	Decrease levels of aspartate aminotransferase and alanine aminotransferase, hence normalizing liver function of patients with hepatitis B. To be noted: results based on a sample of four patients thus larger and controlled studies are required to confirm the effects Hypoglycemic activity, antiviral and liver protective effects in chronic hepatitis B (*in vivo*). To be noted: authors indicated despite pharmacological activities, clinical proof is lacking	Hsu et al., 2008a; Zhou et al., 2005
Antiviral (poliomyelitis)	Polysaccharides	*Agaricus brasiliensis* (previously *Agaricus blazei* ss Heinem.)	Antiviral activity when added during poliovirus infection: potentially acting at the initial stage of viral replication	Faccin et al., 2007
Antiviral (HPV)	Fruit bodies biomass powders	*Trametes versicolor* *Ganoderma lucidum*	Preliminary clinical trial involving 61 male and female patients tested positive for oral human papillomavirus demonstrated 88% clearance of HPV (for both HPV16 and HPV18 serotypes) in a group taking *T. versicolor* and *G. lucidum* capsules for a period of two months. *T. versicolor* and *G. lucidum* proved to be efficacious against HPV. Note: another mushroom – *Laetiporus sulphureus*, which was used in the study as a control, showed no anti-virus properties	Donatini, 2014
Antiviral (herpes zoster)	Hot water extracts of herbal formula containing *Ganoderma lucidum*	*Ganoderma lucidum* 0.75 g/dose (Herbal ingredients included: *Wisteria floribunda* 0.38, *Trapa natans* 0.38, *Miristica agrans* 0.38, *Coix lachryma-jobi* 0.75, *Elfuinga applanata* 0.38, *Panax ginseng* 0.3, and *Punica granatum* 0.38 g/dose)	Hot water extract worked as a pain relief for herpes zoster associated pain in five patients suffering from shingles of different localization and severity. None of the patients developed post-herpetic neuralgia after more than one year of follow-up. Note: this was a small-scale pilot clinical trial, thus, further investigations on larger groups of patients are necessary	Hijikata et al., 2005

Myelodysplastic syndromes (MDS)	Hot water and ethanol extract of whole mushroom	*Grifola frondosa* Maitake mushroom	Phase II clinical trial on the effects of Maitake mushroom showed increased endogenous (basal) neutrophil and monocyte function. Pre-treatment monocyte response to *E. coli* was reduced in MDS patients compared with HC and increased after treatment. fMLP-stimulated ROS production response also increased. The consumption of Maitake β-glucan improves neutrophil and monocyte function in lower-risk MDS patients. The enhanced ROS response to *E. coli ex vivo* in response to treatment suggests that Maitake extract may enhance immune responses against bacterial infection in MDS patients	Wesa et al., 2015
Respiratory (asthma)	Unspecified bioactive extract	*Cordyceps* (unspecified variety)	Inhibit proliferation and differentiation of Th2 cells and reduce the expression of cytokines by down-regulating the expression of GATA-3 mRNA and up-regulating the expression of Foxp3 mRNA in peripheral blood mononuclear cells. Alleviate chronic allergic inflammation by increasing the level of interleukin-10	Sun et al., 2010
Respiratory (silicosis)	JinShuiBao capsules (fermented mushroom mycelia, Cs-4 strain)	*Cordyceps sinensis*	Three JinShuiBao capsules taken three times a day for 2–3 courses (each course = 6 weeks) in addition to conventional therapy showed significant relief in respiratory symptoms (cough, expectoration, chest pain, shortness of breath) compared to the control. Also, treatment group demonstrated significantly lower TNF-α and TGF-β(1) levels, along with higher forced vital capacity, forced expiratory volume in one second, and maximum mid-expiratory flow than before treatment and the control group, suggesting that JinShuiBao has a good therapeutic efficacy in the treatment of silicosis	Miao et al., 2012
Constipation	Fiber	*Auricularia* sp. (ear mushrooms)	Randomized, double-blind trial on 34 enrolled patients showed that fiber supplements made from ear mushrooms improve constipation related symptoms without serious side effects both alone and with additives (although in some cases this combination can cause abdominal discomfort or pain)	Kim et al., 2004

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Table 22.2 (Continued)

Effect/Disease	Bioactive Ingredient or Extract	Species Name	Registered Positive Effects	Reference
Improvement of quality of life	β-glucan	*Agaricus brasiliensis* KA21	Open-label clinical trial conducted on 24 male and female subjects, who have been consuming food with *A. brasiliensis* KA21 for 12-week period, revealed significant improvements in multiple scores depicting subjective perception of life quality (hair loss, amount of gray hair, fatigue, and general malaise, eye strain, shoulder stiffness, cold extremities, difficulty staying awake during the day, and ease of getting out of bed). It was speculated that these improvements might be due to immunomodulating effect of the mushroom	Motoi et al., 2015
Anti-aging (oxygen free radical elimination)	JinShuiBao capsules (fermented mushroom mycelia, Cs-4 strain)	*Cordyceps sinensis*	Three months of treatment with JinShuiBao (three 330 mg capsules, 3 times a day) showed an improvement in some symptoms of asthenia syndrome (Xu-Zheng), including lassitude of loins and legs, intolerance to cold, dizziness, tinnitus, etc., in a group of older men and women (aged 60–84 years). Also, increased levels of antioxidant SOD in red blood cells and decreased levels of MDA in plasma were registered. Note: Barret (2004) indicates that there have been some methodological inadequacies in the study and the therapeutic benefit of the medication remained under question	Zhang et al., 1995
Improvement in exercise performance	Cs-4® (multi-component formulation of *Cordyceps*)	*Cordyceps sinensis*	Clinical trial executed in a double-blind, placebo-controlled fashion demonstrated that 12 weeks administration of Cs-4 increased metabolic threshold (above which lactate accumulates) by 10.5% and ventilatory threshold (above which unbuffered H^+ stimulates ventilation) by 8.5% compared to the control. Changes in vo_2 max were not registered in either of groups. The study suggested that supplementation with Cs-4 improves exercise performance and might contribute to wellness in healthy humans. Note: the data presented by Parcell et al. (2004) showed that 5 weeks of CordyMax Cs-4 supplementation had no effect on aerobic capacity or endurance exercise performance in endurance-trained male cyclists	Chen et al., 2010

a) The references provided by Roupas et al. (2012) are not supplied in the reference list of the current review. In order to view them, please check the original table from the mentioned article.

22.8.4 Dosage Consideration

Defining effective and safe dosages for various herbal and mushroom DS and medicinal preparations represents fairly complicated issues. Dosage of the substance not only determines therapeutic effects of medical treatment along with manifestation and duration of the health benefits, but also directly influences the severity of adverse effects, toxicity, and probability of different complications connected with the treatment. It is difficult to standardize dosages due to the fact that there are many different species of mushrooms possessing medicinal properties and multiple active substances are synthesized by them. Also, these MMs are used in various forms: extracts, powdered fruiting bodies; single mushroom species in the preparation or combination of several mushrooms and/or herbs. Consequently, the concentrations of active ingredients will vary significantly from product to product. There are many other important factors that should be taken into consideration when deciding a dose of the drug, they include – age of the patient, gender, body weight, ethnicity, liver and kidney function, presence of chronic diseases, previous treatment history, whether the patient smokes, and so on. Additional medications prescribed may also affect the drug dose. Thus, it is important to study interactions between MMs preparations and other medicinal substances in order to understand whether they are synergistically amplifying or weakening each other's effects, and whether can they be safely used together in the first place. Apart from that, various individual factors may contribute to this picture making it even more of a puzzle. Folk medicine in different cultures is based mostly on experience accumulated by a number of generations. Because it was developed in times when people knew little about anatomy, physiology, biochemistry, and the whole idea behind a valid scientific approach, this knowledge should be treated with caution. The dose-response relationships of many substances used in traditional medicine are not established, along with their safety, and efficacy. Nowadays we should follow the principles of evidence-based medicine and conduct thorough preclinical-clinical studies of multiple mushroom-derived preparations to receive objective information and create guidelines for the dosage regimen. Additional discussion on dosages of MMs DS is presented in Section 22.5.3.

22.8.5 Pharmacokinetic Issues

Pharmacokinetics is a branch of pharmacology that investigates "movement" of substances administered into living organisms over a period of time. The complex scheme of interactions between the organism and the test substance is commonly divided into the following stages: absorption, distribution, metabolization, and excretion. Sometimes an initial stage called liberation is distinguished. Liberation represents a process of release of a drug from the pharmaceutical formulation. Time-course of the substance concentrations in different systems of the body constitutes the basis of its therapeutic and toxicological effects. Thus, pharmacokinetic studies are usually carried out in parallel with studies of pharmacodynamics that reveal biochemical and physiological properties of the drug. Thorough preclinical and clinical pharmacokinetic studies are mandatory for all synthetic drugs and are executed in order to assess the toxicity of the drug candidate, establish therapeutic schedules, calculate dosage adjustments in particular patients (which may be necessary due to the genetic pharmacokinetic variations). However, data on pharmacokinetics for most herbal and mushroom preparations are fragmented and insufficient.

Recent advances in physiologically-based pharmacokinetic modeling and other novel methodological approaches give scientists a powerful tool that will help make accurate predictions of pharmacokinetic properties of drugs in humans based on preclinical animal studies (Mager et al., 2009). This will allow clinical investigators to reduce the risk exposure of test subjects and patients during the first phases of clinical studies of novel drugs.

The question of bioavailability of different polysaccharides – the major class of biologically active substances derived from MMs is of particular interest, because most mushroom preparations are administered orally. In this sense, bioavailability characterizes the fraction of the ingested dose that will be absorbed after oral administration, presenting one of the principal pharmacokinetic properties of herb/mushroom medicinal substances. Bioavailability is directly connected with dosage related issues.

22.8.6 Preclinical-Clinical Scaling

The development pathway of novel drugs is typically divided into three major stages: discovery, preclinical studies, and clinical trials. Thus, preclinical development represents a very important stepping stone between the initial drug discovery and following human clinical trials. During the course of preclinical studies researchers investigate pharmacokinetics and pharmacodynamics of the drug, select its best formulation, develop future clinical trials design, address the issues of initial starting doses of tested substances in clinical trials, and so on. Ultimately, preclinical studies are aiming to reveal if the new medicinal substance has the potential to enter a clinical phase. Preclinical investigations are required to receive authorities' approval for *in vivo* studies on human subjects, which is why the questions of safety and tolerability of discovered/developed drugs represents one of the major objectives of such studies.

Rigorous preclinical studies including studies on at least two species of animals are mandatory for all synthetic drugs (Zhou et al., 2005). Both rodent and nonrodent mammalian models are used to determine pharmacokinetic patterns and toxicity, as well as to establish general safety of novel drugs (Steinmetz and Spack, 2009). Despite obvious inter-species differences between humans and other mammals proper pharmacokinetic and pharmacodynamic consistent patterns and methods of modeling enable researchers to effectively extrapolate obtained data on efficacy and safety to humans before entering phase I clinical trials. Such scaling procedures are generally reasonable and accepted.

However, one should still be particularly cautious when extrapolating preclinical data on herbal and mushroom substances to clinical studies on humans. For instance, multiple studies *in vitro* and *in vivo* on animals demonstrated that some medicinal mushroom substances, especially polysaccharides (including β-glucans) are activating different factors of immune response, such as macrophages, T lymphocytes, NK cells, induce the synthesis of cytokines, TNF-α, ILs, and IFNs, and so on. However, these significant beneficial effects may not be observed in cancer patients (Zhou et al., 2005). That is why preclinical studies alone do not give the final answer about the efficacy of a drug/preparation on human beings.

22.8.7 Toxicity Issues

Toxicity can be defined as the degree of damage a substance can cause to an organism. Toxicological effects can apply either to a whole organism (animal, plant, bacterium, etc.) or to its target constitutional components, such as cells in multicellular organisms (cytotoxicity), or some specific organs/systems (for instance, hepatotoxicity, neurotoxicity, nephrotoxicity, cardiotoxicity, hematologic toxicity, etc.). A central concept of toxicology points out that these damaging effects are dose-dependent, which makes even virtually harmless substances toxic at some concentrations. Toxicity of herbal medicines is increasingly perceived as an important concern. The field of herbal toxicology is growing in parallel with increasing use of herbal products. Many plants, in the course of their evolution, acquired the ability to produce toxic secondary metabolites, as part of their natural defense mechanisms and survival strategies. Toxicity of plant material to the human body may be caused by toxic constituents and metabolites, unfavorable cellular responses induced by them, or undesirable interactions between

herbal preparations and other administered drugs. A number of studies have demonstrated that some herbal medicines can cause substantial injuries to human organs (Zhou et al., 2005). Another important issue regarding toxicity of some herbs and mushrooms is their ability to accumulate toxic minerals and heavy metals in doses potentially harmful to humans. Long-term consumption of such products leads to the accumulation of these compounds and can cause acute poisoning and severe damage to organs and the whole body. Also, it is known that mushrooms can significantly accumulate different radioactive compounds, when growing in radioactively exposed areas, thus consumption of these mushrooms override all positive therapeutic effects.

Preclinical toxicity studies have demonstrated that many well-known and commonly used herbs possess multiple potential toxicological activities, including ginseng (*Panax ginseng* roots), St. John's wort (*Hypericum perforatum* aerial parts), Kava kava (*Piper methysticum* roots), Ginkgo (*Ginkgo biloba* leaves), Aloe (*Aloe vera* leaves), Licorice (*Glycyrrhiza glabra*), Aristolochia (*Aristolochia contorta*), Willow bark (*Salix* spp.), Germander (*Teucrium chamaedrys*), and many others (Ifeoma and Oluwakanyinsola, 2013; Tewari et al., 2014).

Thus, it is highly important to access possible toxicity of medicinal mushroom products, especially for situations of long-term therapeutic usage or in conjunction with different types of synthetic drugs.

22.9 Conclusions

1) The role of polysaccharide-protein or peptide complexes in pharmacological activity of MMs requires further investigation.
2) More studies are needed to demonstrate which mushroom extracts or compounds are the most effective for specific ailments, including viral infections, bacterial infections, metabolic syndromes, cancer, high cholesterol level, and so on.
3) The development of new methods and processes in the study of MMs must be a priority. For example, a new method was developed in 2009 for nanoparticle extraction of water-soluble ß-glucans from MMs by the Park group from South Korea (Park et al., 2009). A novel process for nanoparticle extraction of Sparan, the ß-D-glucan from *Sparassis crispa*, and Phellian, the ß-D-glucan from *Phellinus linteus*, was investigated using insoluble tungsten carbide as a model for nanoknife technology. This was the first report showing that the nanoknife method results in high yields of Sparan (70.2%) and Phellin (65.2%) with an average particle size of 150 and 390 nm, respectively. The Park group proposed the nanoknife method to be used in producing ß-glucans for food, cosmetics, and pharmaceutical industries. German scientists (Nitschke et al., 2011) developed a new colorimetric method to quantify ß-1,3-1,6-glucans in comparison with total ß-1,3-glucans and a method to quantify chitin in edible mushrooms.
4) The discovery of new species of mushrooms with pharmaceutical activities must be our priority. Only in the last few years, scientists have found new mushroom species with pharmacological activity, for example, *Marasmius oreades*, *Trametes ochracea*, *Xylaria nigrepes*, *Pseudotrameres* (=*Daedalea*) *gibbosa*, *Geastrum saccatum*, *Cyathus striatus*, and *C. olla* (Petrova et al., 2009; Khamaisie et al., 2011; Sharvit et al., 2012).
5) Despite wide use of MMs, their positive effects have been insufficiently studied in human clinical trials. High-quality long-term double-blind placebo-controlled clinical studies of MMs, including well-sized population studies to yield statistical power showing efficacy and safety are definitely needed. Aims of clinical trials during phase I, II, III, and IV must be to obtain sufficient data on efficacy and safety of MM drugs and preparations.

6) More attention must be paid to the studies of MMs on farm animals. On the one hand, there are research areas that could potentially be advanced by using farm animals as biomedical models including obesity, diabetes, aging, cardiovascular diseases, infectious diseases, neurobiology, cancer, nutrition, immunology, ophthalmology, and reproduction. On the other hand, we can revolutionize farm animal research, which is now in crisis (Roberts et al., 2009), by proposing new types of food and DS, antibiotic replacements, and antiviral agents for farm animals.
7) We should draw more attention to research on MMs in the field of plant protection, due to the fact that MMs possess insecticidal, fungicidal, bactericidal, nematocidal, and antiphytoviral activities.
8) Protecting intellectual properties (IP) of MM genetic resources for invention and innovation is a problem that needs more attention. Mushroom genetic resources are currently being utilized and exploited by the pharmaceutical, cosmetic, agricultural, food, enzyme, chemical, and waste-treatment industries. Nevertheless, the role of IP advantages in today's knowledge-driven enterprises is frequently overlooked, despite their potential as sources of monetary value and financial gain. Intellectual properties are often under-managed or under-leveraged. The challenge is how to create, protect, and extract value from IP assets for invention and innovation (Jong, 2005; Wasser, 2010).
9) We must continue to educate society and consumers on MMs science. It is our responsibility as scientists to do much more in educating the public at large on the health benefits of MMs. Advancements made in this scientific discipline are not always visible or available to the public. It is hard to believe that up till now many people all over the world are completely unaware of the health benefits of MMs.
10) It is to our advantage to bridge the gap between Western and Eastern medicine. Western and Eastern medicines have adopted different regulatory systems for herbal and mushroom preparations. Most Western countries follow the rules of the WHO and DSHEA in which plant or MM extracts are defined as DSs, and clinical studies are not required before DSs are introduced to the market. China and several other Asian countries define many of the same herbs and some MMs as drugs, and therefore clinical studies are needed. Western medicine has made little use of medicinal mushroom products partly due to their complex structure and lack of acceptable pharmacological purity. Our target for the future should be to adopt those regulations, standards, and practices from Western and Eastern medicine (Chang and Wasser, 2012; Wasser, 2010).

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Figure 4.5 Crop of DMR-03, a single spore isolate, at a commercial unit.

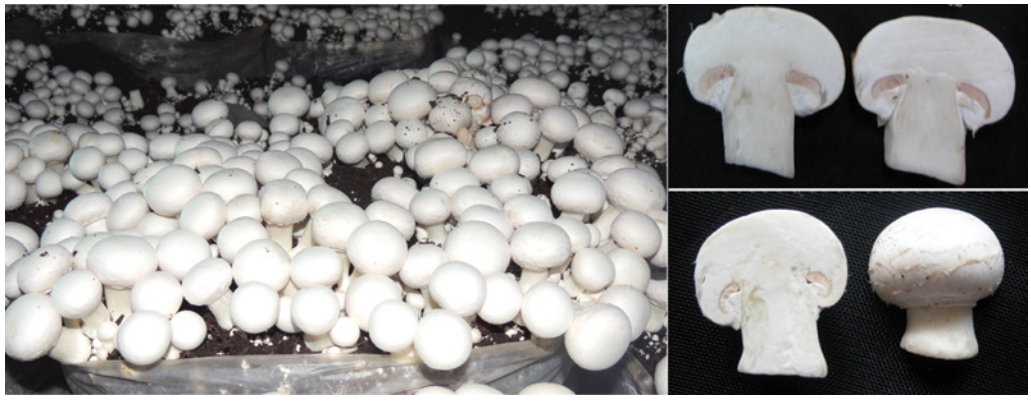

Figure 4.7 Crop of NBS-1 (left), cross section of fruiting body (top right), and quality of fruiting bodies after storage at room temperature for 48 h (bottom right).

Figure 4.8 Crop of NBS-5 (left), cross section of fruiting body (top right), and quality of fruiting bodies after storage at room temperature for 48 h (bottom right).

Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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Figure 4.9 Variability in stipe, pileus, gills and gill cavity in different accessions of button mushroom.

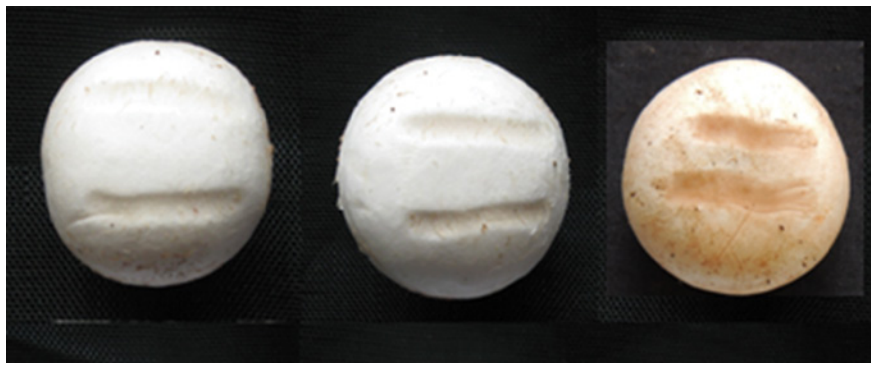


Figure 4.10 Variation in browning after 2 h.

Figure 8.15 Equipment used for irrigation of the cover layer, called the "water tree."

Figure 10.5 Detail of irrigation (indicated by the pale arrow) and ruffling (indicated by the dark arrow).

Figure 11.1 Sciarid flies, adult. Credit: Photo graciously provided by F.J. Gea and M.J. Navarro (CIES, Quintanar del Rey, Cuenca, Spain).

Figure 11.2 Cecid larvae, orange cecid larvae on a mushroom. Credit: Photo graciously provided by Oscar Lahmann.

Figure 11.6 Phorid larvae.

Figure 12.5 *Mycogone perniciosa* on the surface of developed mushrooms. Amber droplets of liquid can be seen on the diseased mushrooms.

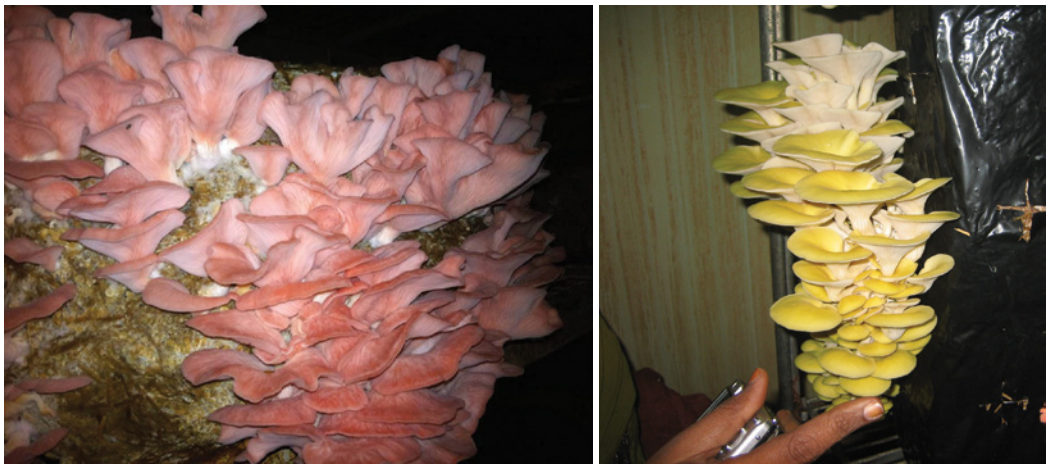
Figure 12.6 Cobweb mycelium (*Cladobotryum mycophilum*) on the surface of the casing and attacking mushrooms (*Agaricus bisporus*).

Figure 12.9 *Trichoderma aggressivum* colonizing compost.

Figure 12.11 Mycelium and ascocarps of *Diehliomyces microsporus* on the casing surface.

Figure 12.13 White plaster mold on the compost surface.

Figure 12.14 Brown plaster mold on the casing surface.



(a) (b)

Figure 14.10 Pink oyster mushroom (a) and yellow oyster mushroom (b).

Figure 15.8 Large autoclaves for sterilization of substrate.

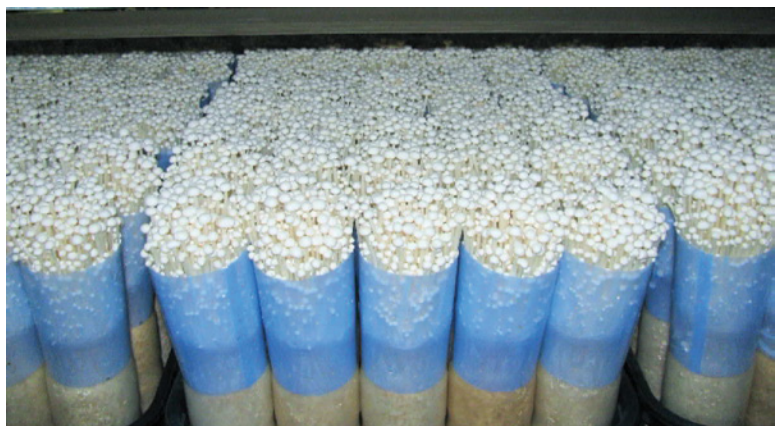


Figure 15.14 *F. velutipes* fruiting in bottle cultivation.

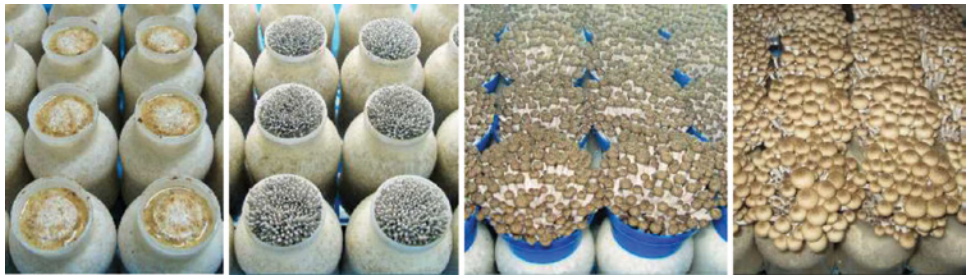


Figure 15.16 Fruit body development of *H. marmoreus* after scratching.

Figure 15.18 *P. eryngii* fruiting in bottle cultivation under LED illumination.

Figure 15.20 *P. nameko* fruiting in bottle cultivation.

Figure 15.22 *G. frondosa* fruiting on colonized substrate in bag cultivation.

Figure 15.23 *L. edodes* fruiting on colonized substrate removed from bags.

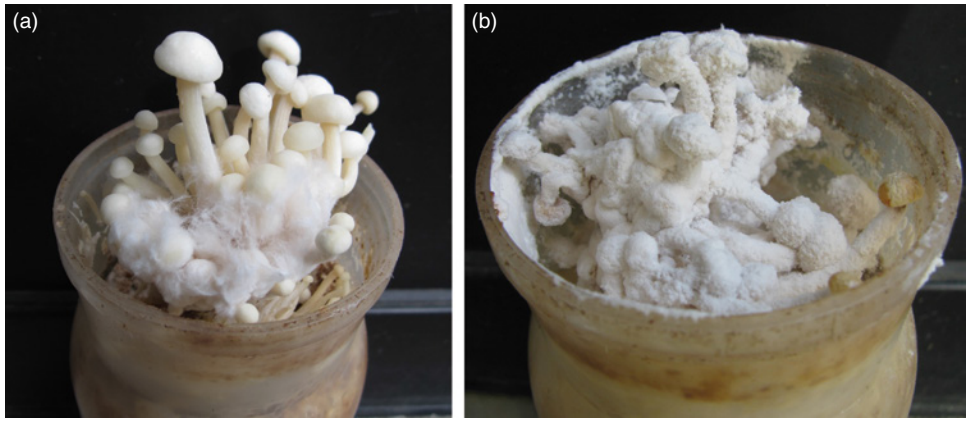


Figure 15.24 Fruit bodies of *Flammulina velutipes* affected by *Cladobotryum varium*. Mushrooms covered with the fluffy mycelium in early stage (a) and enveloped with soft powdery mycelium at a later stage of disease development (b).

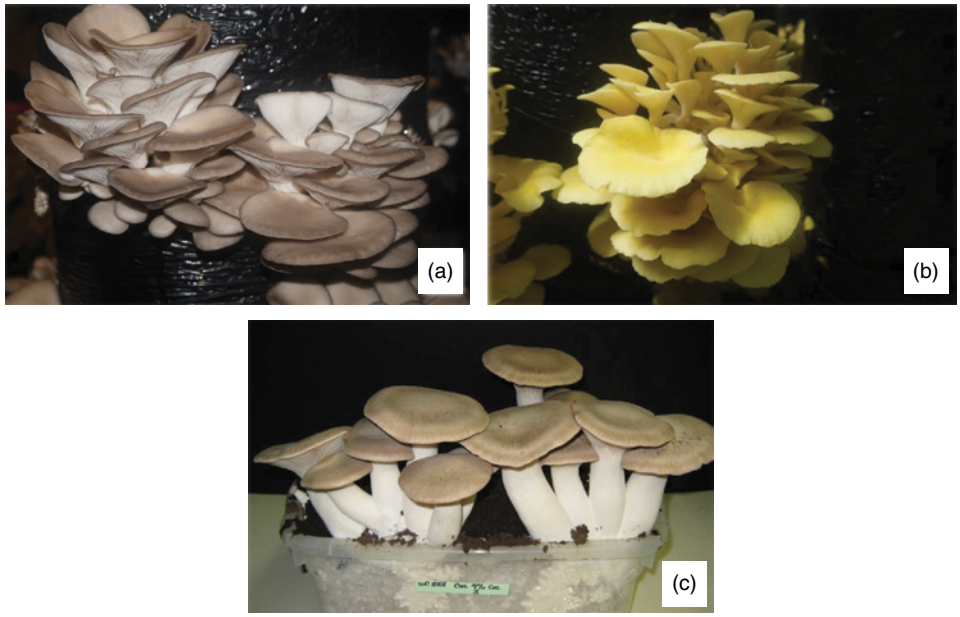


Figure 16.1 (a) Cluster of *Pleurotus ostreatus* basidiomata. (b**)** *Pleurotus cornucopiae* var. *"citrino-pileatus,"* the golden oyster mushroom. (c) *Pleurotus eryngii* growing on cottonseed hulls substrate.

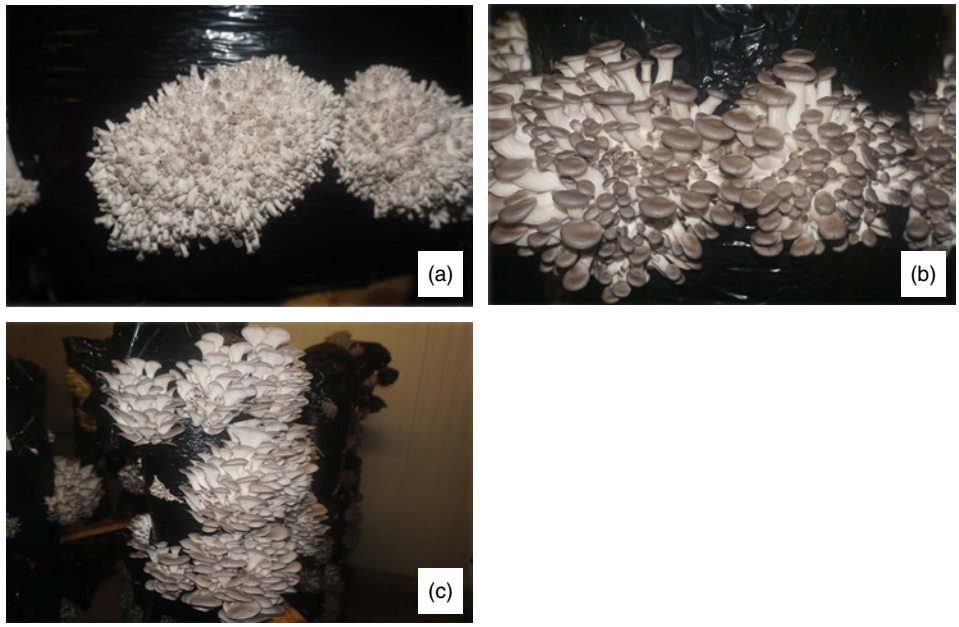


Figure 16.7 (a) Heavy cluster of oyster mushrooms' primordia. (b) Young oyster mushrooms growing on cottonseed hulls/wheat straw substrate. (c) Mature oyster mushrooms growing on cottonseed hulls/wheat straw substrate.

Figure 17.1 (a) Morphological aspects of the mushrooms, (b) spore print, and (c) elliptical spore.

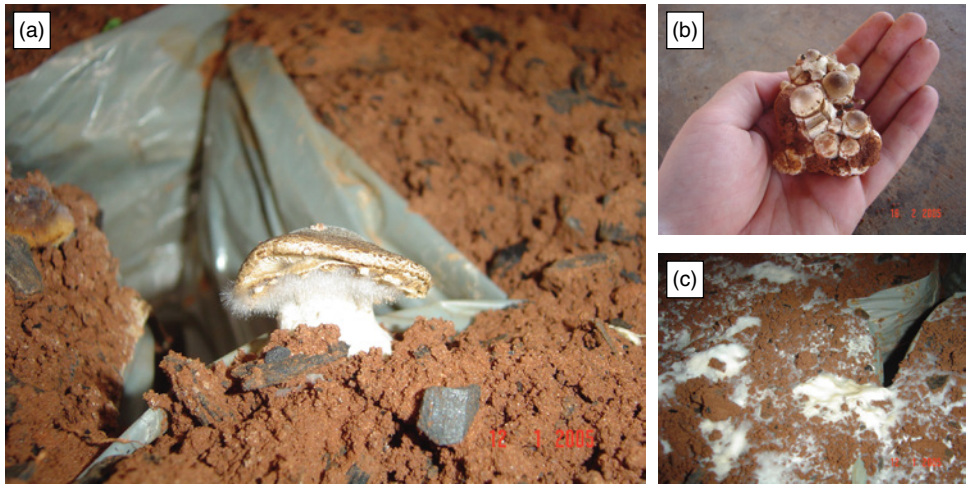


Figure 17.15 (a) Mushroom infected with the mycoparasite *S. megalocarpus*, where it is possible to see its mycelium on lamellae of the Sun mushroom; (b) mushrooms presenting cracks on stipe and brown coloration in the pileus; (c) mycelium of *S. megalocarpus* in the casing layer.

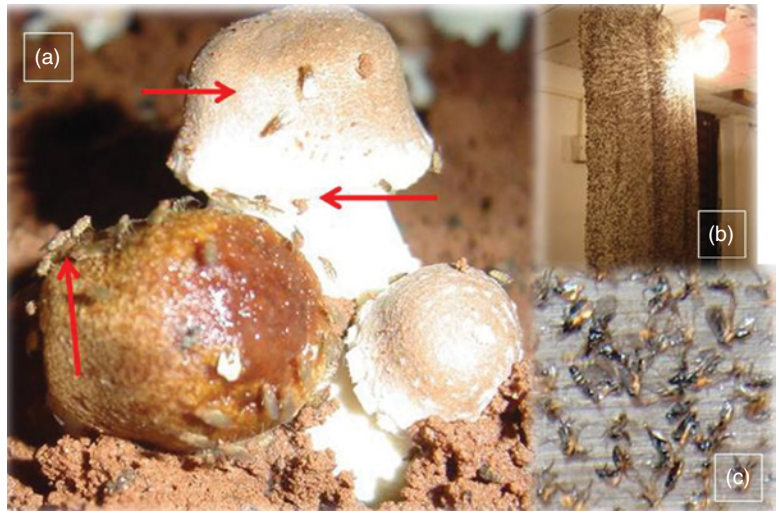


Figure 17.16 (a) Flies in the mushroom, (b) light trap entomological impregnated tail, and (c) close view of the trap with dead flies.

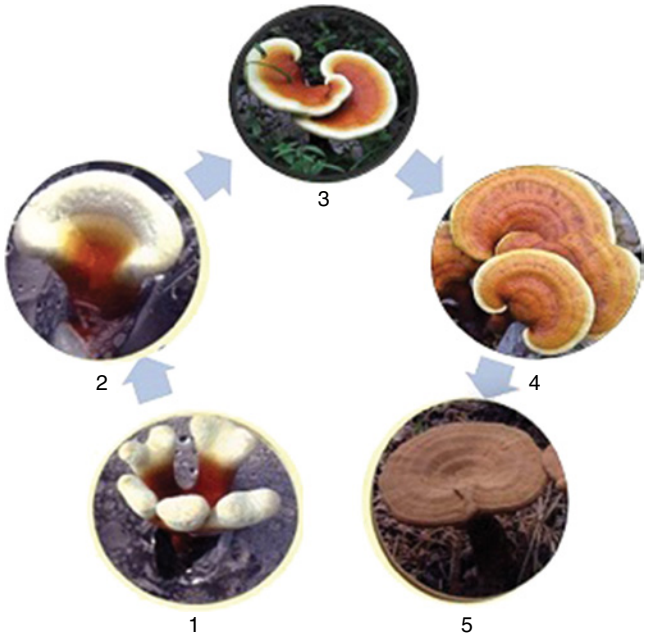


Figure 18.6 Schematic diagram of development process of Lingzhi fruiting body. (a) Bud-breaking stage; (b) Bud-developing stage; (c) Developing stage; (d) Growth stage; (e) Maturity stage. *Source:* Zhou 2012. Reproduced with permission of Springer.

Figure 19.3 Production of *Polyporus tenuiculus* in bags.

Figure 19.5 Production of *Agrocybe cylindracea* in supplemented wheat straw.

Figure 19.7 Production of *Gymnopilus pampeanus* in bags.

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Edible and Medicinal Mushrooms: Technology and Applications, First Edition.
Edited by Diego Cunha Zied and Arturo Pardo-Giménez.
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